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(54) GENE PROTEIN PRODUCT AND TREATMENT OF CELL

(57)Abstract:

PURPOSE: To obtain a substantially purified, loss-free and biochemically active protein having high potency by introducing a genetic substance into an insect cell cultured product and culturing the produced genetic protein.
CONSTITUTION: A genetic substance is introduced through a virus vector or the like into an insect cell cultured product to produce a gene protein. The gene protein product obtained by culturing the gene protein functions at cellular level or subcellular level when introduced into a target cell.

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CLAIMS

[Claim(s)]

[Claim 1] In the approach of producing the gene production polypeptide related to a predetermined gene refined substantially Choose the vector which conveys cDNA which has the nucleotide sequence which is substantially equivalent to a nucleotide sequence in this gene, insert this DNA in this vector, and this vector is introduced into a cell culture object. The polypeptide production approach which consists of forming the manifestation system which spreads this polypeptide, growing up this cell, disuniting this cell, and extracting this polypeptide from this cell, and refining this polypeptide.

[Claim 2] The approach according to claim 1 this vector is a virus.

[Claim 3] The approach according to claim 1 this virus is a baculovirus.

[Claim 4] This virus is Autographa. The approach according to claim 1 of being a californica nuclear polyhedrosis virus.

[Claim 5] The approach according to claim 1 this cDNA has a PORIHE drine compounds gene promotor property.

[Claim 6] The approach according to claim 1 of this cDNA having the promotor of a PORIHE drine compounds gene, and inserting this cloning cDNA in this promotor's lower stream of a river.

[Claim 7] The approach according to claim 1 this cell is an eukaryote cell.

[Claim 8] This cell is Spodoptera. The approach according to claim 1 of being a frugiperda cell.

[Claim 9] The method according to claim 1 of carrying out this purification by the immunity affinity chromatography method.

[Claim 10] The method according to claim 1 of guiding this cDNA from an eukaryote gene.

[Claim 11] The method according to claim 1 of guiding this cDNA from an eukaryote antioncogene.

[Claim 12] The method according to claim 1 of guiding this cDNA from a retinoblastoma gene.

[Claim 13] The polypeptide manifestation system which consists of a vector for conveyance, and a cell culture object in the polypeptide manifestation system which discovers the gene production polypeptide related to a predetermined gene.

[Claim 14] The manifestation system according to claim 13 this whose vector is a virus.

[Claim 15] The manifestation system according to claim 13 this whose vector is a baculovirus.

[Claim 16] This vector is Autographa. System according to claim 1 which is a californica nuclear polyhedrosis virus.

[Claim 17] The system according to claim 13 whose cell of this cell culture object is an insect cell.

[Claim 18] This cell is Spodoptera. System according to claim 13 which is a frugiperda cell.

[Claim 19] With regards to a predetermined gene, choose the vector which conveys cDNA which has the nucleotide sequence which is substantially equivalent to a nucleotide sequence in this gene, insert this DNA in this vector, and this vector is introduced into a cell culture object. The polypeptide which the manifestation system which spreads this polypeptide is formed, this cell was grown up, and this cell was disuniting, and extracted this polypeptide from this cell, and was obtained by the approach of consisting of refining this polypeptide and which was refined substantially.

[Claim 20] A polypeptide including guiding this cDNA from a retinoblastoma gene according to claim 19.

[Claim 21] The therapy approach which consists of checking this deficit, a mutation gene, or that there is no gene in the approach of having a deficit antioncogene and a mutation antioncogene or treating a cell without a gene, and asking for an opposite part without the deficit which is the natural student gene or its clone of this gene, and producing the protein production object of the gene of an opposite part.

[Claim 22] An approach including this conveyance carrying out the microinjection of this protein production object

to this cell according to claim 21.

[Claim 23] The approach according to claim 21 this purification includes the dialysis to the buffer solution of this protein.

[Claim 24] This buffer solution is tris. - Approach containing HCl, KCl, EDTA, DTT, and glycerol according to claim 21.

[Claim 25] This tris - Approach according to claim 24 pH of HCl is 7.4.

[Claim 26] This buffer solution is KCl of DTT of the one section, EDTA of the one section, and the 100 sections, and tris of the 200 sections. - Approach containing HCl according to claim 23.

[Claim 27] An approach given in claim 21 term in which this check investigates the existence of a protein production object, and measures the amount of protein production.

[Claim 28] An approach including determining whether this measurement uses a specific antibody for this protein, and this antibody and the immune complex of this protein form according to claim 21.

[Claim 29] The drugs constituent which serves as a polypeptide as an active ingredient from suitable support physiologically.

[Claim 30] The drugs constituent according to claim 29 this whose polypeptide is pp110RB.

[Claim 31] The drugs constituent according to claim 29 which chooses this active ingredient from the protein production object of a milk antioncogene, a Wilms' tumor inhibitor, a Beckwith-Wiedemann syndrome inhibitor, a transition sexual cell neoplasm inhibitor, a neuroblastoma inhibitor, a small cell lung cancer inhibitor, a kidney androblastoma inhibitor, and a colorectal cancer inhibitor.

[Claim 32] A drugs constituent with the following amino acid sequences of this active ingredient according to claim 29.

[Formula 1]

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Industrial Application] On the whole, this invention relates to the cell therapy for treating the production approach of a gene protein production object, and a cell, and controlling tumorigenesis. This invention relates to the cell therapy method which controls a cell therapy and tumorigenesis again. This invention is government support (license number EY05758) invention, and is invention attained in collaboration with national American Insurance Association (the National Institute of Health) and a cull FORUNIA university. Therefore, about this invention, the government also has a fixed right.

[0002]

[Description of the Prior Art] There is research quite detailed about the tissue of Homo sapiens or an animal, and much deficits are checked on cell level. Many of these deficits have a hereditary cause, and most depends it on a deficit gene. A deficit may consider point mutation etc. as a cause, and as a result, the nucleotide sequence of the gene itself may divide, or it may show an unusual change. When there is a malfunction gene, the gene protein production object with which it becomes impossible to have produced the gene protein production object finally, and itself suffered a loss will be produced.

[0003] A gene therapy can be applied when identifying a deficit gene about both Homo sapiens and an animal. In this case, what is necessary is to lead to the nucleus of the cell which should treat a cloning gene, and just to correct an unusual genetic material or a deficit genetic material. The matter used for this approach has a high manufacturing cost, and needs a complicated elaborate experimental device, and needs application of a complicated elaborate genetic manipulation technique. Generally such a technique cannot be used, therefore only the laboratory where most treats molecule genetic manipulation comparatively elaborate to a small number of altitude can be used.

[0004] Costs of a gene therapy are high, and since it generally cannot use, another cell therapy is searched for. It is possible to draw a conclusion from analysis of the molecular structure and the function of a protein production object about the insurance of the gene which produces this production object. For example, what is necessary is to use a gene protein production object in many cases, and just to opt for the interaction between genes, in order to determine the neoplasm control mechanism of the body. What is necessary is just to refer to the parent-patent application specification of this application about a neoplasm control mechanism. the top where it is reliable and cost is low in order to solve a gene function and a gene interaction — dependability — the approach of producing the gene protein production object which can offer a lot of [possible / prediction] high protein is very desirable.

[0005] Not only a gene protein production object is useful, but [although a gene function and a gene interaction are solved] this protein itself can use it for the therapy of a deficit gene condition. In this case, it is advantageous to introduce the suitable gene protein production object for a cell with a deficit genetic material, and it is effective. Cost is low and sometimes easier than the case where the direction of installation of a protein production object prescribes the genetic material itself for the patient for the purpose of a therapy by the case.

[0006] What is necessary is just to refer to the patent application specification submitted to this application and coincidence about a protein therapy.

[0007] When it sees from the recognition about the importance of a gene protein production object, it is very advantageous that the approach of preparing and separating a gene protein production object in the form refined substantially can be used now. in order that that the activity matter can be used as it is so much biochemically may study the biochemical property and molecule behavior which are participating in the heredity mechanism — a therapy sake — an owner — it is a meaningless advance.

[0008] Generally, in production of a different laboratory scale from the case of extensive production, the gene protein production object is produced also by the synthetic production only from the cell. Speaking of the induction from a cell, little existence of the cell protein is recognized very much. Consequently, the attempt which guides sufficiently a lot of protein from the source of nature is not realistic.

[0009] Speaking of the synthetic producing method, the attempt which introduces the decode array of a gene into a bacteria expression vector, and discovers protein was not successful except for some cases. Bacteria production protein has weak solubility. The point of giving the result which a bacterial cell could not change eukaryote protein, and analysis of such protein mistook when modification after a translation was required about protein has another fault which uses a bacteria manifestation system. That is, generally bacteria production protein has low solubility, and since there is a molecule deficit, usefulness has a limit.

[0010] It not only cannot produce enough a lot of protein, but by the usual laboratory-approach which makes a protein production object, the purity of production protein is not enough, either and variation is in purity. TrpE-RB fusion protein may be discovered for the example of representation of the difficulty at the time of producing a certain kind of protein production object, T7 RNA polymerase discovered to E.coli may be used for it, and a polypeptide may be produced. These approaches are comparatively complicated and need complicated elaborate biochemical actuation. Furthermore, since the target polypeptide does not carry out little deer production very much, these approaches have a big limit. Moreover, the polypeptide produced by this approach is seen in molecule, and it is common not to be desirable. For example, it is because this is not phosphoryl-ized.

[0011] Therefore, biochemical [desired] and the desired approach of producing a polypeptide with a biophysical property in a substantial amount are strongly desired from the importance of a gene production object polypeptide.

[0012] A remarkable advance is seen about an understanding of the function of the gene in living body health maintenance. If that it can generally say cannot produce protein with proper cytogene, various kinds of abnormalities will occur to a living body. It can ask that the whole gene is lost or the gene itself is missing by various kinds of causes for this reason that cannot be produced. From recognition of these factors, remarkable progress is accepted in a gene therapy.

[0013] For example, it is thought that the Wilms' tumor which is infantile kidney cancer is produced by gene inactivation of a chromosome 11. It is proved that oncogenicity can be controlled, if the normal chromosome 11 is introduced into a Wilms' tumor cell when the minute cell fusion medium transferring method of a single strand chromosome is used. On the other hand, this effectiveness is not accepted in Chromosome X and installation of 13.

[0014] On experiment level, although a certain extent of transition of a human chromosome without a deficit is useful, such transition is not applicable to the therapy of a genetic defect. Preparation of the suitable chromosome for a therapy not only needs remarkable skill, but requires time amount for one upwards, and it is because cost is high. For this reason, in many cases, this approach is nonpermissible.

[0015] When it is not desirable to use a chromosome without a deficit for a therapy, thinking theoretically below is introducing into a patient the proper whole gene or the part which has the effect at least. Although this approach has workability higher than the approach of introducing a chromosome without a deficit, it is only a certain fixed case that a gene therapy is desired.

[0016] Not only cost is very high, but separation of the proper nucleic-acid matter, sequencing, and cloning require time amount about this point. In addition, the complicated elaborate molecule genetic manipulation approach which can be used only in the location restricted very much also in the world is required of such an approach. Furthermore, such an approach produces a lot of matter suitable for a therapy even now.

[0017] As mentioned above, a specific therapy of the cell level which cost is comparatively low, and it is reliable, and can more generally use by using the bionics-approach, and uses the specific matter in a biochemical operation is desired strongly. Furthermore, a production object effective in a therapy is introduced on cell level, and a therapy which brings about change of neoplasm control etc. is also desired strongly. Although it is needless to say, it is strongly wished also about the production object which can produce so much in the state of purification without variation, and can be introduced into a deficit cell easily and effectively.

[0018] It is in the 1st purpose of this invention offering a specific therapy safe on the whole and and a production object effective in controlling cancer control.

[0019] It is in another purpose of this invention offering a cancer inhibitory-control production object and a therapy effective in the control and extermination using the bionics-approach and a production object of a cancer

tumor. Moreover, it is also the purpose of this invention to offer the drugs constituent for cancer treatment which demonstrates an operation on cell level and the level between cells.

[0020] It is in still more nearly another purpose of this invention offering the drugs constituent which treats the symptom produced by the absence of the deficit or mutation antioncogene which makes nature or a synthetic production object an active ingredient, and an antioncogene.

[0021] This invention leads a specific antioncogene protein production object to an object cell, and consists of a cell therapy which realizes cancer control.

[0022] This invention offers the conventional radiotherapy and the cure for cancer with little need for a chemotherapy. Furthermore, this invention approach can be applied after discovery of disposition which is easy to suffer from cancer hereditarily at the very early stage before tumorigenesis begins.

[0023] this invention — an antioncogene protein production object — good facilities — and in the point of using it at comparatively low cost and realizing cancer control on cell level, it is very advantageous.

[0024] Although there is a point which has not been clearly carried out about whether cancer will be generated enough when the antioncogene beyond one or it in a certain cell inactivates then, cell installation of a gene protein production object is new approach, and is the advantageous approach to a malignant tumor therapy. This invention is in the condition which suppressed the damage to a living body to the minimum unlike the cytotoxic cancer therapy from the former, and is still more advantageous in the point of bringing about an advantageous change on cell level.

[0025] As mentioned above, it is in the 1st purpose of this invention offering the approach of producing an activity gene production polypeptide without the deficit of a substantial amount.

[0026] Furthermore, another purpose of this invention has structure and a function in offering the same production approach of a specific gene production polypeptide as a natural student polypeptide.

[0027] If it summarizes, the above-mentioned purpose and other purposes of this invention are realizable by the approach of producing a substantial quantity of a request polypeptide by introducing a genetic material into an insect cell. For example, if a cloning gene, gene fragmentation, or its derivative is introduced into a host cell using a suitable vector, it is a substantial amount and the protein of a high grade can be produced with a high level.

[0028] This invention produces the polypeptide of high quality in a substantial amount, offers the approach of carrying out things of investigating a gene function on cell level, and is very advantageous.

[0029] Moreover, this invention is advantageous, is reliable, moreover, can produce this polypeptide at repetitive comparatively low cost, and is very advantageous also in this point.

[0030] Furthermore, this invention can produce the high quality polypeptide of a considerable amount which can solve an intergenic interaction on cell level and the level between cells, and is advantageous also at this point.

[0031]

[Easy explanation of a drawing]

Other purpose, descriptions, and these implementation approaches should become clear from the following publications at the above-mentioned above-mentioned purpose of this invention and the description, and the list. Moreover, it refers to an understanding of this invention itself at an accompanying drawing, and it should be obtained if the publication about the example of invention is read. Drawing 1 is the schematic diagram showing the configuration of the Baculoviridae manifestation vector which compounds pp110RB. Drawing 2 is the western blotting (Western blot) of a ppRB infection insect cell. Drawing 3 is western blotting which shows the cell extract extracted from the infected cell even 72 hours after infection. Drawing 4 is a microphotography in which the normal position between cells of RB protein is shown. Drawing 5 is the microphotography of infection Sf9 cell. Drawing 6 is a radioautograph which shows the result of phosphoryl-izing of RB protein in an insect cell, and dephosphorylation analysis. drawing 7 — pMG 3-245 — anti- — it is drawing showing the rough lysate from -RB, infection Sf9 cell, and the electrophoresis analysis result of eluate. Drawing 8 shows the fusion protein and the south Western (Southwestern) DNA binding measuring method of Baculoviridae-manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10%. It is the radioautograph of the blot from parallel gel to the gel used in order that drawing 9 might create drawing 8, and the incubation of this blot is carried out by the 32P-labeling DNA fragment. It is the chromatogram which shows complexing of drawing 10 Baculoviridae-manifestation RB protein and an SV40 T antigen. The nuclear transformation of the purification RB protein after the microinjection to the cytoplasm of drawing 11 Saos-2 cell is shown.

[0032]

[The best example of invention] All have adopted the contents here and the reference which makes reference on

these specifications constitutes a part of this specification publication. Hereafter, this invention will be explained in the following order.

A. an outline — the nuclear transformation B7. epitome [0033] of specific complex formation B6. purification RB protein with the RB protein purification B5. DNA-avidity from the phosphorylated B4. infection insect cell after the nuclear normal position of the manifestation B3. extraversion RB protein of the extraversion RB protein in the configuration B-2. infection insect cell of example of B. RB gene production object B1. recombination Baculoviridae, and a translation, and an SV40 T antigen A. When outline this invention introduces a genetic material into an insect cell culture object, it can be used for this gene protein producing a specific gene protein production object by culture about the approach of producing gene protein. If this approach is used, the deficit with a high force value refined substantially can twist, and activity protein can be obtained biochemically. [0034] The manifestation system of this invention has a large application. That is, for example, Homo sapiens, the cloning gene of an animal, its fragmentation, a homologue, a derivative, or a part is applicable to production of a request protein production object. Thus, needless to say, the produced protein is applicable to the therapy of a deficit cell at the elucidation of a gene function. a flume — obtaining — it is because a gene interacts on cell level.

[0035] Since the insect cell culture object had an eukaryote property when a gene protein production object was produced, the suitable thing was found out. In order to introduce a genetic material into a cell culture object, the usual vectors, such as a virus vector, can be used.

[0036] For example, in order to produce RB gene protein production object, the virus vector of an insect cell culture object is used. In order to rearrange with a high level in a culture insect cell and to produce protein about this point, Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.

[0037] This invention is a cell therapy which introduces a specific antioncogene protein production object into an object cell, and realizes neoplasm control. Since a given cell has a deficit gene, in the point which causes the protein manifestation deficit in a cell, it may be missing.

[0038] It is related with this invention approach using the gene protein production object related to a deficit gene. A purification protein production object is introduced into an object cell, for example, neoplasm control is realized. By supporting and introducing this production object into support suitable in pharmacology, this protein production object is cell level, or acts on the bottom level of a cell.

[0039] the cell RB gene protein which whose RB gene which is an antioncogene is missing as an example of this approach, or does not have it was boiled and introduced. What is necessary is just to refer to the parent-patent application specification which has made reference on these specifications about the detailed information about RB gene.

[0040] In the suitable example, the small-child cancer which is a retinoblastoma, i.e., a symptom with the rare retina under growth, as a PM of recessive tumorigenesis research is used. Based on the normal position and its recessive nature of the heredity element to a chromosome 13q14 which is involving, the gene presumed to be an antioncogene, i.e., a retinoblastoma susceptibility gene, (RB) was cloned. A 4.7 kilobase mRNA imprint is discovered at all in the normal organization which the exon of 27 distributed this gene to the genomic DNA of 200 kilobases, and inspected. By the sequence analysis of a complementary DNA clone, the long open reading frame which has coded the hypothetical protein of the amino acid of 928 became clear. When the antibody to the predetermined epitope guessed from the RBcDNA array was used, as for this RB gene production object, relative molecular weight (Mr) was identified the nucleus phosphoprotein of 110,000–114,000. This was named pp110RB.

[0041] only to a retinoblastoma, it has become clear that the deficit of RB gene function is boiled also at the neoplasm of some of others including a breast cancer, an osteosarcoma, a prostatic cancer, or small cell lung cancer again, and is involving. According to the latest research, if RB gene is reintroduced into a retinoblastoma, an osteosarcoma, or a prostate gland cancer cell by retrovirus medium gene transition, it will have become clear

that some fields of new phenotypes including the tumorigenesis in a nude mouse are controlled clearly. This is a direct proof of the neoplasm control function of RB gene. However, the foundation in the molecular level of this biological activity is not established yet.

[0042] Although all the biochemical properties and biological functions of an antioncogene production object are not solved the place to current, it is because there is little this protein in a cell as for this, so it is difficult to obtain in sufficient amount. What is necessary is just to refer to the parent application specification (the name of invention: the production approach of a gene protein production object) for which it applied to this application and coincidence about this point.

[0043] The cell therapy which has a deficit gene or introduces a specific gene protein production object into a cell without a gene was invented. According to this invention, the deficit refined substantially twists and the proper amount of activity gene production protein can be biochemically introduced into a deficit gene with an effective dose on a therapy.

[0044] The cell therapy of this invention has a large application application. This protein production object is useful not only to the therapy of a deficit cell but the elucidation of a gene function. It is because a gene interacts on cell level.

[0045] In the one specific example, a retinoblastoma gene protein production object, i.e., pp110RB, has a deficit gene, or it can apply it to the therapy of an eukaryote cell without a gene widely.

[0046] In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed the SV40 T antigen and the specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0047] B. Explain in more detail about the example of RB gene production, next the production approach by this invention of pp110RB protein. What is necessary is just to refer to the following parent-patent application specifications about information detailed to the pan about this protein and a retinoblastoma gene.

[0048] Although all the biochemical property and biological functions of an antioncogene production object, such as RB gene, are not solved, this is because it is difficult to obtain purification protein in sufficient amount. That is, it is because it is because a cell has little protein and the protein manifestation by the installation to the bacteria expression vector of the coding array of a gene has not succeeded in one except for a part in addition again. It has been concluded that the problem which the approach used now has from these was avoidable if a cloning gene is discovered in an eukaryote system. Although it will be an antioncogene if this invention is illustrated, although the specific example of this invention is related with production of RB gene protein production object, it can apply also to production of the protein production object of other eukaryote genes which are not restricted to this, therefore is related also with production of such a protein production object.

[0049] In order to produce recombination protein with a high level in a culture insect cell, it is desirable although Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.

[0050] In order to test the manifestation possibility of functional RB protein by the Baculoviridae system, cloning Homo sapiens RBcDNA including the perfect coding array of RB stirp was introduced into the AcNPV expression vector, and the recombination virus was bred in the insect cell. The Homo sapiens pp110RB manifestation was realizable with a high level with the host-vector system. The obtained protein was phosphoryl-ized and the nucleus of an infected cell was correctly made into the target. Furthermore, the purification method of RB protein was also established. In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed an SV40 T antigen and specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0051] In order to produce RB protein in the configuration Baculoviridae manifestation system of B1. recombination Baculoviridae to the maximum extent, it rearranged by removing a great portion of 5' non-coding

array from RB gene, and the transition vector was constituted. By seat singularity mutagenesis, two BamH1 seats were introduced into NUKURECHIODO 116 and 2935 of RBcDNA, and the configuration of a recombination transition vector was promoted. PORIHE drine compounds 5' a non-coding array with refined pAcYM1/RB2.8 [perfect (60 base pairs) as shown in drawing 1] -- 5' of RBcDNA -- it unites with a non-translated field and nRAN which a perfect coding array follows after that is coded. This recombination gene does not contain an ATG codon for the upstream of NUKURECHIODO 139 of Shinsei RB initiation seat. Therefore, a recombination gene codes un-uniting and full length RB protein.

[0052] Although the transition vector pAcYM1 is shown in drawing 1, these all have the upper array of the PORIHE drine compounds gene containing A of an initiation ATG codon, and BamH1 seat characteristic next continues. There is a publication of Matsuura etc. about a transition vector. J. Gen.Virol., 68:1233-1250 (1987). pRB 44-2 includes the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning in BamH1 seat of the plasma pGEM1 (Promega). About the recombination Baculoviridae vector, pAcYM1/RB2.8 [i.e.,], since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed in the bottom of direct control of a polyhedrin promotor.

[0053] The following points were taken into consideration, when a Baculoviridae manifestation vector was constituted and pp110RB was compounded. pRB 44-2 becomes BamH1 seat of pGEM1 from the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning. Including EcoR1 fragmentation of about 7 kbs of a virus DNA array located in the side face of a PORIHE drine compounds gene, in this case, pAcYM1 permutes all the PORIHE drine compounds coding arrays except the 1st A of ATG by BamH1 linker, although the leader sequence remains as it is. Since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed about a recombination Baculoviridae vector including polyhedrin promotor RBcDNA fusion, pAcYM1/RB2.8 [i.e.,], in the bottom of direct control of a polyhedrin promotor. The array of a fusion joint is shown in the direction under drawing 1. Moreover, the case notation under drawing 1 shows a polyhedrin promotor, and the upper case notation shows a RBcDNA array. And BamH1 linker is underlined. An arrow head shows the translation of the fusion gene using ATG (nucleotide 139) of RB. a* (+1) of drawing 1 shows the 1st A of the translation initiation codon ATG of a PORIHE drine compounds gene.

[0054] The imprint to a viral genome from the recombination plasmid of RBcDNA is a wild type Autographa by the RIPOFE cushion (lipofection). It carried out by carrying out the conte run SUFE cushion (contransfection) of the PaCym1/RB2.8DNA using a californica nuclear polyhedrosis virus DNA (BRL). Since a PORIHE drine compounds closing object was not accepted in an infected cell, the recombination virus which inactivated the PORIHE drine compounds gene by the ARERIKKU permutation with RB gene by family recombination was identified according to the plaque gestalt. Plaque purification of the virus was carried out 3 times, and the pure Baculoviridae stock was obtained. This was set to AcNPV-Y4RB.

[0055] Before the manifestation AcNPV polyhedrin promotor of the exogenous RB protein in B-2. infection insect cell determines whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell Sf9 cell is prepared. The yeast rate of 3.33 gm/l (yeastolate), insect culture-medium (Grace's insect medium)-Bull.1555 (a Texas (1987) agricultural experiment place --) of the grace which added a lactalbumin hydrolysis production object (GIBCO) and 10% of heating inactivation fetal calf serum (GIMINI), A college experiment place and TX are used and it is Sf9, i.e., Spodoptera, by the monolayer or suspension culture at 27 degrees C. frugiperda IPLB-Sf21-AE [In vitro, 13:213-217 (1977)] was cultivated. When carrying out large-scale production of the cell lysate, the spinner culture of Sf9 cell was cultivated by the EX-CELL400 convention culture medium (J. R.Scientific) which does not contain a blood serum. Suspension culture of the malt (Molt) -4 cell of Homo sapiens T leukemia cell lineage was carried out by RPMI1640 culture medium which added the calf serum 20%. Saos-2 cell of osteosarcoma cell lineage was cultivated by the Dulbecco (Dulbecco) denaturation Eagle's medium which added fetal calf serum 7.5%.

[0056] When an AcNPV PORIHE drine compounds prow motor determined whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell, infection processing of the Sf9 cell was carried out by plaque purification AcNPV-Y4RB. 40 hours after infection, the lysate of an infected cell was collected and immunoprecipitation processing was carried out with anti--RBO-47 antigen. Next, after carrying out SDS-PAGE processing of the sample, western blotting analysis was carried out.

[0057] The pp110RB identification result in the AcNPV-Y4RB infection insect cell performed to drawing 2 and

drawing 3 by western blotting analysis is shown. In the case of drawing 2, the cell extract was prepared 40 hours after infection from the simulation infected cell (path 2), the AcNPV-Y4RB infected cell (path 3), or wild type AcNPV infection Sf9 cell (path 4). The malt -4 of Homo sapiens leukemia cell lineage was used as contrast (path 1).

[0058] Although the manifestation was accepted in the extract (path 3) of an AcNPV-Y4RB infected cell in RB protein of the same full length as a mammalian cell (path 1) when immunity blot processing was carried out by the monoclonal system antibody as shown in drawing 3, in the case of the simulation infected cell or the wild type AcNPV infected cell (paths 2 and 4), it did not accept. In order to ask for the optimal stage of RB protein production, in the case of drawing 3, the time amount after infection was changed, and the cell extract was obtained from AcNPV-Y4 infected cell in it. Immunoprecipitation processing of the lysate was carried out with anti-RB0.47 antigen, and immunity blot processing was carried out by the pMG3-245 monoclonal system antibody. p110RB of drawing 3 and pp110RB express non-phosphorylated protein and phosphorylated protein, respectively. After infection acted as the monitor of the production of RB protein, and asked for the optimal stage to collect cells. As shown in drawing 3, RB protein production could be detected 24 hours after infection, and the 12 next hours increased the amount of production considerably. Protein production level was maintained all the time over about 72 hours after infection, and the bacteriolysis of a cell started it substantially 72 hours after. In order to suppress protein degradation accompanying lysis to the minimum, infected cells were usually collected about 40 hours after infection.

[0059] When detecting the manifestation of RB protein, AcNPV-Y4RB was used and infection processing of the Sf9 cell was carried out by MOI of 0.5. The cell of 5×10^4 was dissolved 24, 36, 48, 60, and 72 hours after from infection in the 1ml dissolution buffer solution (Naf [of Nonidet (Nonidet) P-40;1mM / of EDTA;100mM] of NaCl;50mM of NaCl of 50mM, and 7.4; 0.2% of pH, and PMSF of 1mM), respectively, at long-intervals alignment processing was carried out for 5 minutes, and the lysate was defecated. Next, the incubation of the lysate was carried out with anti-RB0.47 antigen, and the immune precipitate was separated by SDS-PAGE 7.5%. Then, protein was moved to nitrocellulose paper and it processed with the conventional method. After leaving it in an overnight cut off state and carrying out incubation processing of the nitrocellulose paper with pMG3-245 anti-RB monoclonal system antigen for 3 hours, according to Cell and the publication of 54:275-283 (1988), it processed by the alkaline phosphatase junction goat anti-mouse IgG and the chromophoric substrate.

[0060] As for the phosphorylated RB gene after the nuclear normal position of B3. exogenous RB protein, and a translation, molecular weight (Mr) codes the nucleus phosphoprotein of 110,000. In order to investigate whether RB protein produced into the insect cell by Baculoviridae reached the nucleus correctly, 40 hours after infection, anti-RB0.47 antigen was used and immunity dyeing of the AcNPV-Y4RB-infection Sf9 cell was carried out. The normal position between cells of RB protein discovered into the insect cell by immunity dyeing is shown in drawing 4 and drawing 5. The case of AcNPV-Y4RB-infection Sf9 cell is shown in the case of mock infection Sf9 cell, and drawing 5 at drawing 4. As shown in drawing 4 and drawing 5, the infected cell contained the unusually large nucleus. Such a condition is peculiar to the cytopathogenic effect of the Baculoviridae infection. Dyeing was not accepted when the incubation of mock infection or the wild type AcNPV infection Sf9 cell was carried out with anti-RB0.47 antigen (drawing 4). However, dyeing strong only against the nucleus of an AcNPV-Y4RB infected cell was accepted (drawing 5). When SDS-PAGE and Western blotting analyzed the nucleus and cytoplasm extract from AcNPV-Y4RB infection Sf9 cell, it has checked that exogenous RB protein mainly existed in a nucleus part.

[0061] Immunity dyeing was performed at the following process. Also in both mock infection wild type AcNPV infection and AcNPV-Y4 infection the overnight incubation of the Sf9 cell was inoculated and carried out to the chamber slide (Miles Scientific) which carried out coating of the Polyl-L-lysine (Sigma) 40 hours after infection. Before moving from all to the following process, the phosphoric-acid buffer sodium chloride water solution washed the slide. That is, the interstitial cell was fixed for 20 minutes for 10 minutes using the acetone (-20 degrees C) using the 0.04M phosphate buffer solution of 4% formaldehyde of ****. After carrying out the pre-incubation of the fixed cell for 10 minutes with the PBS solution of 2% of normal goat blood serum, the overnight incubation was carried out with anti-RB0.47 antigen diluted to 0.02% of triton X-100. The BIOCHINIRU-ized goat anti-rabbit IgG (Canada and TAGO of Burlingame) was added after washing. 1 hour after, after carrying out the incubation of the interstitial cell for 45 minutes using AB complex joined by the horseradish peroxidase (Canada and Vector Labs of Burlingame), the incubation was carried out using the following substrate. This substrate consisted of 0.05% of 3 and 3'-diaminobenzidine tetrahydro chloride, and 0.01% of a 0.05M tris-HCl solution of H₂O₂, pH7.6 (Sigma). The

reaction was suspended by carrying out PBS washing of the cell after 3 – 5 minutes. Next, a photograph of a cell was taken with the NIKON diamond photograph microscope (diaphotomicroscope).

[0062] The result of phosphoryl-izing of RB protein produced into the insect cell at drawing 6 and dephosphorylation is shown. 40 hours after AcNPV-Y4RB infection, a 35S-methionine or 32P-orthophosphoric acid was used, and metabolic turnover labeling of the Sf9 cell was carried out in 3 hours. Malt -4 was used as contrast and immunoprecipitation of the cell lysate was carried out using anti-RB0.47 antigen. before carrying out potato acid phosphatase (PAP) processing, (paths 1, 2, 3, and 4) were separated, SDS-PAGE (path 1', 2', 3', and 4') separated 35S- and a 32P-labeling RB protein immune complex after processing, and radioautography analyzed. Similarly, the dephosphorylation experiment was conducted using the lysate from a non-labeled cell, and western blotting analysis was performed before and after potato acid phosphatase processing (paths 5 and 6 and 5', 6').

[0063] Furthermore, the case of drawing 4 is explained continuously. Phosphoryl-ization of RB protein is produced in a multiplex serine and threonine residue, and the molecular weight heterogeneity of RB protein in SDS-PAGE is explained. In order to investigate whether phosphoryl-ization is received after RB protein produced into [Oncogene Res., 1:205-214(1989);Cell, 56:57-65 (1989)] insect cell translating, a 35S-methionine or 32P-orthophosphoric acid was used from 3 hours after infection in 40 hours, and metabolic turnover labeling of the AcNPV-Y4RB-infection Sf9 cell was carried out. Immunoprecipitation processing of the cell extract was carried out, and radioautography processing was carried out after carrying out SDS-PAGE analysis. What is necessary is just to refer to the paths 2 and 4 of drawing 6 about this point, respectively. It was parallel to this, the immunoprecipitation nature RB protein from the same extract was processed by potato acid phosphatase (PAP), and the dephosphorylation effectiveness RB protein migratory [in SDS-PAGE] was tested. The molecular weight 110,000 of 35S-labeling RB protein fell from the double band to 1-fold band after dephosphorylation (path 2' of drawing 6). And activity emitted almost completely from 32P-labeling RB protein (path 4' of drawing 6). The band change pattern as PAP processing also with the same dephosphorylation analysis by Western blotting about the lysate from a non-labeled cell which carried out infection processing by AcNPV-Y4RB was shown (the path 6 of drawing 6 , 6'). These observation result showed having phosphoryl-ized RB protein produced into the insect cell. Moreover, the molecular weight heterogeneity of RB protein accepted by SDS-PAGE from this change has been explained.

[0064] Radiation labeling and dephosphorylation analysis of Sf9 insect cell were carried out at the process shown below. 40 hours after infection, the incubation of the Sf9 during 30 minutes cell (3×10^6) was carried out using the 30mm pan by the methionine and the DME culture medium containing neither of a phosphoric acid which added 10% of fetal calf serum. Then, metabolic turnover labeling of the cell was carried out by adding a 0.25 mCi(s)/a ml 35S-methionine (1134 Ci/mole, NEN) or 0.25 mCi(s)/ml 32P-orthophosphoric acid (support nothing, ICN) in 3 hours. The cell was extracted using the dissolution buffer solution (tris of 50mM – NaF [of Nonidet P-40;1mM / of EDTA;100mM] of NaCl;50mM of HCl and 7.4; 0.2% of pH, and PMSF of 1mM), and immunoprecipitation processing of the extract was carried out with anti-RB0.47 antigen.

[0065] Potato acid phosphatase (PAP and Boehringer) dephosphorylation analysis of two thirds of the immunoprecipitation RB protein from 35S- or the 32P-labeling cell lysate, and the non-labeled cell lysate was carried out. [Oncogene Res., 1:205-214 (1989)]. The incubation of the RB protein content immune complex was carried out using PAP of 1.5 units in 37 degrees C (leupeptin of MES of 20mM, and 2; 50micro [of NaCl;1MgCl(s) of mM of pH5.5;100mM] M) of reaction buffer solutions, and 60 minutes, and after the reaction, after carrying out SDS-PAGE analysis 7.5%, radioautography or Western blotting analyzed RB protein.

[0066] Infection processing of the Sf9 cell was carried out by AcNPV-Y4RB by the multiplicity of infection (MOI) of purification 1.0 of RB protein from B4. infection insect cell, and the cell lysate was obtained 40 hours after infection. The total level of RB protein discovered in the Baculoviridae system on this condition was about 17–18mg per 1l. of infection insect cell culture objects ("109 cell). About this point, **** which illuminates Table 1 3 is good.

[0067]

[Table 1]

Table [] 1 purification process of the recombination RB protein from the Baculoviridae infection insect cell. Sum total tongue RB tongue Yield Whenever [purification] Purity Park mass Park mass (%) (%) (mg) (mg) A cell extract 670a 16c 90c 1.0 times 2.3pMG3-245 immunity affinity column 13.5b 12.9d 72 cds 41.3 times 95 Protein quantum c. western blotting by the protein quantum b. micro BCA (PIERCE) by the approach and spectrophotometry of a. Bradford (Bradford) (Bio-RAD) And the protein quantum by the protein quantum d.

Coomassie-brilliant-blue-staining method by the density measurement method, and the density measurement method [0068] As shown in Table 1, 90% of discovered RB protein (16mg) was accepted in the supernatant after cell division, and 10% had stopped at the insoluble fraction. Since 2.3% of all cell protein was formed, RB protein was easily detectable from the cell lysate. About 135mg protein was recoverable from the alkaline eluate of a column after one-step immunity affinity chromatography purification. In order to investigate the purity of elution RB protein, SDS-PAGE and Coomassie brilliant blue staining analyzed the eluate aliquot equivalent to 2.5×10^5 cell.

[0069] What is necessary is just to refer to drawing 7 which shows immunity affinity chromatography purification of pp110RB about this point. After analyzing the aliquot (equivalent to 2.5×10^5 infected cell) of eluate with the electrophoresis method by 10%SDS-poly AKURIRO amide gel from the rough lysate from mock infection Sf9 cell (path 1) or AcNPV-Y4RB infection Sf9 cell (path 2) of 1×10^5 , and pMG3-245 anti-RB immunity affinity chromatography, it analyzed by the Coomassie-brilliant-blue-staining method. The arrow head has shown RB protein of anticipation molecular weight.

[0070] When judged by the density measurement method, the above-mentioned one-step purification process is effective, and RB protein was able to be obtained by 41.3 times whenever [95% / of purity / (drawing 7 , path 3), 72% / of yield /, and purification] (Table 1).

[0071] Some modification was added although the immunity affinity column was constituted according to the publication of Shneider, etc. ****, etc. [J. Biol.Chem., 257:10766-10769(1982);Virology, 144:88-100(1985)]. After filling up the Bio-Rad (Bio-Rad) column with 2ml protein G-agarose (Genex) and washing by 0.01-N HCl, the joint buffer solution (sodium acetate of 0.1M, NaCl of pH5.0;0.1M) washed. It joined together by adding the anti-fRB monoclonal antigen (pMG 3-245) of 2mg [15] to a column. Next, the boric acid buffer solution of 0.1M washed the column widely, and the bead was re-suspended in the 20ml buffer solution. Dimethyl PIMERIMI date dihydrochloride (Sigma) was added so that the last concentration might be set to 40mM(s), mixture was stirred at the room temperature for 1 hour, and crosslinking reaction was performed. after washing and a room temperature -- for 10 minutes, 20ml solution of ethanolamine-0.1M boric acid buffer solutions of HCl of 40mM, and pH8.0 -- it came out and the residual reactivity radical of a bead was intercepted. then, the glycine of 0.2M and pH2.3 -- it came out and the column was washed, and it saved in this solution until it neutralized and was needed with tris buffers (tris-HCl of 50mM, EDTA [of pH7.4;100mM] of NaCl;1mM of PMSF:1mM). When OD280 of the first monoclonal antigen sample and OD280 of the flow fraction in the following process were measured, about 10mg pMG 3-245 had combined with the protein G-agarose which is 2ml.

[0072] About RB protein, two biochemical properties are described despite specific complex formation current [with B5.DNA avidity and an SV40 T antigen]. That is, it is one of them that DNA is combinable as a peculiar property, and it is one more [Nature, 329:642-645 (1987)] and that how many kinds of the neoplasm protein and the specific complex of that DNA tumor virus can be formed [Cell, 54:275-283,(1988) Science, 243:934-937 (1989);Nature (London), 334:124-129 (1988)]. About these [which are participating in proteinic biological functions] two well-known biochemical properties, RB protein refined from the Baculoviridae infection insect cell was tested.

[0073] A south Western DNA-joint measuring method is shown in drawing 8 and drawing 9 . The purification trpE-RB fusion protein of 6microg and purification Baculoviridae manifestation pp110RB were applied to SDS-PAGE 10%. In the case of the joint measuring method shown in drawing 8 , the Coomassie-brilliant-blue-staining method was used, and, in the case of the joint measuring method shown in drawing 9 , the electric imprint of the parallel gel was carried out at nitrocellulose paper. Next, the incubation of the blot was carried out by the 32P-labeling DNA fraction, and radioautography analysis was carried out. In the case of drawing 8 and drawing 9 , it is as follows. Path 1:RB19-22; path: -- RB23-27; -- path 3:RB19 -- the purification RB protein from a -27; path 4:AcNPV-Y4RB infection insect cell.

[0074] In the case of drawing 8 and drawing 9 , DNA binding was measured by south Western analysis. In this case, SDS-PAGE separated the trpE-RB fusion protein of tales doses, and the purification RB protein from an insect cell 10%. The amount of the protein by which loading was carried out was calculated by the Coomassie-brilliant-blue-staining method (drawing 8). In the experiment which used another parallel gel, after carrying out the electric imprint, the incubation was carried out to the nitrocellulose membrane by the 32P-labeling DNA. Radioautography analyzed next DNA combined with protein (drawing 9). The affinity to DNA of the fusion protein RB 19-27 with which the main domain interacts with DNA is 20 times as high as two fields RB 19-22 and RB 23-27. About this point, the path 3 of drawing 9 could be compared with paths 1 and 2, and purification full length RB

protein showed strong DNA avidity like RB 19-27 (drawing 9 , path 4). The DNA avidity of the purification RB protein from an insect cell is elution which continues after that from a column even if based on maintenance of the protein by the DNA cellulose. - It has proved also by NaCl- of about 400 mM(s).

[0075] Purification of pp100RB from an infection insect cell was carried out as follows. By MOI of 1.0, using AcNPV-Y4RB, infection processing was carried out and suspension culture of the Sf9 cell was carried out (1X166 cell / ml, 1000ml). 40 hours after infection, low-speed centrifugal separation pelletizes and washes a cell, and it is tris of 50mM(s). - It re-suspended in the extract buffer solution containing NaCl;10% the leupeptin of PMSF;25microg of DTT;1mM and 50 units of NP-40;1mM of glycerol (v/v);1mM of HCl and 7.4; 0.2% of pH / aprotinin of ml. [of EDTA;100mM] After carrying out an incubation for 15 minutes in Hikami, centrifugal (for 10,000xg, 4-degree-C, and 10 minutes) clarification processing of the sample was carried out, and RB content supernatants were collected. As mentioned above, immunity affinity chromatography of pp110RB was carried out in the column of 2ml capacity containing the anti-fRB monoclonal antigen (pMG 3-245) combined with protein G-agarose. After letting a supernatant pass 4 times in a column, the following solution was used in the amount of 200 bed capacity, respectively, and the column was washed continuously. The dissolution buffer solution, the dissolution buffer solution containing NaCl of 500mM, and a penetrant remover (PMSF;10% glycerol [of 200mM / of NaCl;1mM] of EDTA;1mM of DTT;1mM). Next, binding protein was eluted from the column with the alkaline elution buffer solution containing PMSF of EDTA;1mM and 10% of glycerol of NaCl;1mM of the triethylamine of 2mM, and pH10.8;200mM. 1ml fractions are collected and it is tris of 1M immediately. - It neutralized by 1/12 capacity of HCl (pH7.5), and saved at -70 degrees C in 10% glycerol.

[0076] When refining pp110RB from an infection insect cell, after calculating the total amount of protein, the south Western DNA binding measuring method and the SV40 T antigen joint measuring method were enforced.

[0077] The amount of all the protein of the elution fraction of an immunity affinity column was calculated with the micro-BCA measuring method (PIERCE). SDS-PAGE analyzed the elution protein sample next, and the Coomassie-brilliant-blue-staining method and the degree were asked for the amount of RB protein of eluate by the density measurement method. The total amount of protein of a cell extract was measured by the approach (Bio-Rad) of Bradford. [Anal.Biochem., 72:248-254 (1976)]. In order to carry out the quantum of the RB protein of the cell lysate, after using the purification RB protein diluted in order as a criterion and carrying out Western blotting, density measurement compared band strength. What is necessary is just to refer to Table 1 about this point.

[0078] Protein blotting was carried out with the conventional method. According to Bowen's (Bowen) etc. publication, the incubation of the blot was carried out by the radiation labeling DNA. [Nucleic Acids Res.8:1-21 (1980)]. This was performed at the room temperature. After rinsing the blot quickly, it washed 3 times by the urea of 6M, and 0.2% of NP-40 (all are 20 minutes), and washing (all are 30 minutes) was succeedingly carried out 4 times with the DNA binding buffer solution (NaCl;0.2% BSA;0.2% Ficoll400 and 0.2% of polyvinyl pyrrolone of EDTA;50mM of tris-HCl of 10mM, and pH7.0;1mM). Next, the incubation of the blot was carried out for 30 minutes in the DNA binding buffer solution including the 32P-labeling DNA. By EcoR1, line-ized pGEM1DNA was labeled by the alpha-32P deoxy nucleotide (Amersham, >3000 Ci/mmol) by the random priming, and it was used as a probe. Washing of the blot was carried out 3 times (all are 10 minutes) with the DNA binding buffer solution after hybridization, it was air-dry and radioautography analyzed. TrpE-RB fusion protein was included as contrast. Each trpE-RB fusion protein was named according to the exon of RB gene which protein contains. That is, RB 19-22, RB 23-27, and RB 19-27 occupy the pp110RB field of an exon 19-22 (amino acid 612-775), an exon 23-27 (amino acid 776-928), and an exon 19-27 (amino acid 612-928), respectively.

[0079] Immunity chromatography purification of the SV40 T antigen was carried out from Ad-SVX1-infection 293 cell. [J. Virol., 53:1001-1004(1985);Cold Spring Harbor Press., Cold SpringHarbor, NY, pp.187-192(1982)]. And anti-T monoclonal PAG419 antigen was obtained from Oncogene. The well-known complex formation measuring method was enforced. However, some modification was added. That is, the Baculoviridae manifestation RB protein of 800ng was mixed with the 1ml EBD buffer solution (NaCl of tris-HCl of 50mM, and pH8.0;120mM, and 0.5% of Nonidet P-40) containing PMSF of 1mM, 25microg [/ml] leupeptin and 50 units / aprotinin of ml. The purification T of 800ng(s) was added to mixture, and the incubation was carried out in Hikami during 90 minutes. anti- -- using either of -RB0.47 or PAB419 antigens, immunoprecipitation of the aliquot of mixture was carried out and Western-blotting analysis was carried out. The blot made it react with pMG 3-245 and PAB419 in order. After carrying out an incubation with the alkaline phosphatase junction goat *-mouse IgG, coloring processing of the blot was carried out using the color-enhancing substrate.

[0080] In order to test the capacity for purification RB protein to be able to form an SV40 T antigen and specific complex, equivalent RB protein and an equivalent T antigen were mixed, and immunoprecipitation of the aliquot of mixture was carried out using anti--RB0.47 antibody or -anti-T antigen PAB419.

[0081] In relation to this point, the complex which Baculoviridae manifestation RB protein and an SV40 T antigen form in drawing 10 is shown. Purification Baculoviridae manifestation RB protein was mixed with the purification T antigen by in vitro one. next, the mixture of this aliquot -- PAB419 (path 2) -- anti- -- immunoprecipitation was carried out by either of -RB(s)0.47 (path 3), and Western blotting analyzed. Paths 1 and 4 show the purification Baculoviridae manifestation RB protein which carried out immunoprecipitation by the purification SV40 T antigen which carried out immunoprecipitation by PAB419, respectively, and anti--RB0.47 antibody.

[0082] As shown in drawing 10, when the T antigen was mixed with RB protein by in vitro one, PAB419 not only coimmunoprecipitates with RB protein, but (the path 2), and T and anti--RB0.47 antigen coimmunoprecipitated (path 3). It is proved [data / these] that RB protein from the Baculoviridae infection insect cell can form an SV40 T antigen and specific complex.

[0083] Since it became clear that the nuclear transformation purification protein of B6. purification RB protein had the two well-known biochemical properties of RB by in vitro one, it investigated about the in vitro behavior of purification protein next. The cytoplasm of Saos-2 cell with the osteosarcoma cell lineage without an exon 21-27 which codes C terminal cutting RB protein (p95), including a deficit RB gene was injected with purification RB protein. [Proc.Natl.Acad.Sci.U.S.A., 87:6-10 (1990)]. The antibody orientated this protein to the cytoplasm in consideration of the fact of going to the C terminal of RB protein, in the small quantity of extent which is not accepted by anti--RB0.47 antibody to be used. Immediately after injection, the cell was fixed and it analyzed by the immunity staining technique. The nuclear normal position of generation RB protein after carrying out a microinjection to drawing 11 to the cytoplasm of Saos-2 cell is shown. The cell was injected with generation RB protein and it analyzed by the immunity staining technique. The arrow head shows among drawing that dyeing of the nucleus after a microinjection is strong compared with the non-injecting cell.

[0084] As shown in drawing 11, compared with a non-injecting cell, dyeing of the nucleus after a microinjection is strong (arrow head), and it is shown that injection protein was immediately conveyed to the nucleus. since RB protein is known as a nucleoprotein -- the nuclear normal position of after a microinjection and purification protein -- immediately -- and although cut correctly, this has suggested that this protein is activity in in vivo one.

[0085] In the case of the microinjection, purification RB protein was dialyzed so that the last concentration might become [ml] the injection buffer solution containing DTT of EDTA;0.1mM and 2% of glycerol of KCl;0.1mM of tris-HCl of 20mM, and pH7.4;10mM in 0.5mg /. The capillary glass tube needle (Eppendorf) was used and the microinjection of the Saos-2 cell which grew on the glass CHANN bar slide was carried out by the usual approach. The micro manipulator and opposition contrast microscope (NIKON make) of Eppendorf which attached the vacuum pressurizer were used, and micromanipulation of the capillary tube was carried out, respectively, and the microinjection process was visualized. After the microinjection, the cell was immediately fixed with the 0.04M phosphate buffer solution solution (pH7.4) of formaldehyde 4%, and it analyzed by the immunity staining technique.

[0086] As explained more than B6. epitome, it has proved that the bottom Homo sapiens retinoblastoma gene production object of the transcriptional control of the Baculoviridae polyhedrin promotor could be efficiently discovered. Since RB protein had the misgiving which it not only blocks growth of a cell, but shows "toxicity" to it depending on the case, it was thought that the attempt which has discovered RB protein on high level for a long time was difficult. The imprint of the foreign gene from a polyhedrin promotor is produced in the second half of infection, and cutoff of production of an extracellular virus particle, a cell, and a great portion of virogene continues next. Therefore, when superfluous production is carried out, the Baculoviridae insect cell lineage is advantageous to composition of protein, such as RB protein which has that it is harmful to cell growth. Another advantage of this system is in the similarity of an insect and the protein synthesis path of a mammalian cell.

[0087] Moreover, it also becomes clear that RB protein reaches the nucleus of an insect cell correctly, and this connotes that the nuclear normal position signal of mammalian is recognized by the insect cell. Although glycosylation of the recombination protein in the Baculoviridae manifestation system is restricted to 0-association of a high mannose mold, and an N-joint oligosaccharide, suitable phosphoryl-ization of outpatient department protein is reported about c-myc and the manifestation of HTLV-Ip40x (J. Virol.). Although RB protein was phosphoryl-ized from before, not carrying out glycosylation was known. This is a reason for production of functional RB protein with the suitable Baculoviridae manifestation system.

[0088] As indicated on these specifications, RB protein produced into the infection insect cell is phosphoryl-ized.

after a translation, therefore can specialize a multiplex band by Western-blotting analysis completely like the case of RB protein of Shinsei mammalian. However, when seeing from band strength and it compares with hyperphosphorylized RB protein, phosphoryl[non-]-izing and a phosphoryl-ized gestalt are superior. Since this phenomenon's being reflection of the cell cycle condition of the population at the time of viral lytic infection or a lot of exogenous RB exist in a cell the place to current, it is not known whether it is because phosphoryl-izing of the protein by the insect kinase is only inadequate. In order to determine the same thing **** as the case of phosphoryl-ized PATANGA mammalian protein, and accuracy, it is necessary to map the phosphoryl-ized location of RB protein in a precision.

[0089] The total level of the recombination RB protein discovered in the Baculoviridae system is about 17 to 18 mg per 1l. (109 cells) of infection insect cell culture objects. This manifestation level is equivalent to other mammalian protein which this system generates. For example, in the case of interleukin 2, it is 10-20 mg/l [The Banbury Report, Fields, B., Martin, M.A. & Kamley, D. (ed.), 22:319-328 (1985), Cold Spring Harbor Laboratory Press, Cold Spring Harbor]. moreover, [Oncogene, 4:759-766(1989)]. which is 4-5 mg/l in P210 BCR-ABL — if a recombination transition vector including the 5' PORIHE drine compounds 5 without deficit united with RBcDNA which removed most non-coding fields' non-translated field is used, high RB protein manifestation level can be made still higher. This RBmRNA array is G+C Rich highly, and is a factor advantageous to forming stable secondary structure. These structures are considered to make translation effectiveness of Correspondence mRNA low when it is before an initiation codon. about the in vitro translation of RBmRNA, it proves [become / 10 times / high.] from 5 times by permuting RB5' a non-translated array by it of an alfalfa mosaic virus (AMV) RNA 4 or beta globulin mRNA — having — this — RB5' — [EMBO J. (1990)] which is what suggests having a bad influence on the translation of a non-coding array potentially. Moreover, if 5' non-translated array with a long foreign gene exists, it will also have become clear that the recombination PORIHE drine compounds manifestation in the Baculoviridae system is influenced. since A+T rich nature of a polyhedrin promotor is very strong, before inserting in a transition vector as a conclusion — long — and G+C — rich 5' non-coding array should be trimmed from RBcDNA, and the pp110RB manifestation should be optimized.

[0090] In order to suppress the denaturation of the protein under purification to the minimum, several kinds of different protocols were tested about elution of RB protein from an affinity column. Since many are not known so much about the biological functions and the biochemical property of RB protein, it is only two parameters that it can be used as a scale of the integrity of purification protein. That is, it is complex formation with DNA avidity and an SV40 T antigen. In addition, the conditions which maintain a proteinic biochemical property were elution conditions which use the triethylamine of 20mM(s) by pH10.8 in this invention. Although purification protein carried out the nuclear normal position immediately from the cytoplasm after the microinjection, it is proved [this / protein / this] that activity is shown under these elution conditions. When protein was eluted by extreme pH (triethylamine of the glycine of 200mM, pH 2 and 3, or 100mM(s), pH11.5), the inclination for protein to denaturalize was accepted. That is, the two above-mentioned activity fell. This is clear also from insoluble floc having formed after prolonged preservation.

[0091] According to the former report, although [non-phospho RURIRU-ized RB protein] the SV40 T antigen of D2C2 cell which is the stable transformant by the SV40 T antigen of nephrocyte network valve flow coefficient1-P of an ape is combinable, it was found out that the HIPOHOSUHORIRU-ized gestalt of [Cell, 56:57-65 (1989)] and RB protein of a certain kind can also form an SV40 T antigen and complex. This has been checked with sufficient repeatability, when in vitro mixing of the purification RB protein [from a T antigen and an AcNPV-Y4RB infection insect cell] or malt -4 lysate was carried out. The same phenomenon was accepted also when complex was formed by in vivo one using a Cos cell (drawing 10). Since phosphoryl-ization of RB protein is changed in a cell cycle in the case of phase singularity and the complex formation between RB and viral neoplasm protein is participating in the transformation activity of these DNA tumor virus, the relevance of HIPOHOSUHORIRU-ized RB protein and an SV40 T antigen should be solved someday.

[0092] RB protein which does not have a meltable deficit when the Baculoviridae insect cell lineage is used, as explained above and which can also predict activity suddenly — an owner — it is a big advance for future biochemical and research of a biophysical property of RB gene production object that it can be used in a meaningless amount. They are analysis of the cell protein in relation to the case considered, three-dimension-structure research of RB protein using separation and X-ray crystallography of the specific DNA array with which these interact, etc. Moreover, the elucidation of the biological functions of the retinoblastoma gene in cancer control should also progress. About the intervention to the cell growth of RB and differentiation which were

directly tested by the microinjection considered, it is inquiring on the current energy target.

[0093] The notation used on these specifications is explained below. cDNA -- complementary DNA and kd -- KIRODARUTON and kb -- a kilobase and SDS -- for Nonidet P-40 and MES, sodium salt (2-[N-morpholino] ethane sulfonic acid) and MOI are [a sodium dodecyl sulfate and PAGE / polyacrylamide gel electrophoresis and NP-40 / relative molecular weight and PAP of the multiplicity of infection and Mr] potato acid phosphatase. The protein production object shown by "pp110RB" is the same protein production object as "ppRB110."

[0094] It is included by the pneumonia and the range which various kinds of modification is possible and all indicated to the claim although this invention has been explained about the specific example. That is, this indication does not mean a limit.

[Translation done.]

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TECHNICAL FIELD

[Industrial Application] On the whole, this invention relates to the cell therapy for treating the production approach of a gene protein production object, and a cell, and controlling tumorigenesis. This invention relates to the cell therapy method which controls a cell therapy and tumorigenesis again. This invention is government support (license number EY05758) invention, and is invention attained in collaboration with national American Insurance Association (the National Institute of Health) and a cull FORUNIA university. Therefore, about this invention, the government also has a fixed right.

[0002]

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TECHNICAL PROBLEM

[Description of the Prior Art] There is research quite detailed about the tissue of Homo sapiens or an animal, and much deficits are checked on cell level. Many of these deficits have a hereditary cause, and most depends it on a deficit gene. A deficit may consider point mutation etc. as a cause, and as a result, the nucleotide sequence of the gene itself may divide, or it may show an unusual change. When there is a malfunction gene, the gene protein production object with which it becomes impossible to have produced the gene protein production object finally, and itself suffered a loss will be produced.

[0003] A gene therapy can be applied when identifying a deficit gene about both Homo sapiens and an animal. In this case, what is necessary is to lead to the nucleus of the cell which should treat a cloning gene, and just to correct an unusual genetic material or a deficit genetic material. The matter used for this approach has a high manufacturing cost, and needs a complicated elaborate experimental device, and needs application of a complicated elaborate genetic manipulation technique. Generally such a technique cannot be used, therefore only the laboratory where most treats molecule genetic manipulation comparatively elaborate to a small number of altitude can be used.

[0004] Costs of a gene therapy are high, and since it generally cannot use, another cell therapy is searched for. It is possible to draw a conclusion from analysis of the molecular structure and the function of a protein production object about the insurance of the gene which produces this production object. For example, what is necessary is to use a gene protein production object in many cases, and just to opt for the interaction between genes, in order to determine the neoplasm control mechanism of the body. What is necessary is just to refer to the parent-patent application specification of this application about a neoplasm control mechanism. the top where it is reliable and cost is low in order to solve a gene function and a gene interaction -- dependability -- the approach of producing the gene protein production object which can offer a lot of [possible / prediction] high protein is very desirable.

[0005] Not only a gene protein production object is useful, but [although a gene function and a gene interaction are solved] this protein itself can use it for the therapy of a deficit gene condition. In this case, it is advantageous to introduce the suitable gene protein production object for a cell with a deficit genetic material, and it is effective. Cost is low and sometimes easier than the case where the direction of installation of a protein production object prescribes the genetic material itself for the patient for the purpose of a therapy by the case.

[0006] What is necessary is just to refer to the patent application specification submitted to this application and coincidence about a protein therapy.

[0007] When it sees from the recognition about the importance of a gene protein production object, it is very advantageous that the approach of preparing and separating a gene protein production object in the form refined substantially can be used now. in order that that the activity matter can be used as it is so much biochemically may study the biochemical property and molecule behavior which are participating in the heredity mechanism -- a therapy sake -- an owner -- it is a meaningless advance.

[0008] Generally, in production of a different laboratory scale from the case of extensive production, the gene protein production object is produced also by the synthetic production only from the cell. Speaking of the induction from a cell, little existence of the cell protein is recognized very much. Consequently, the attempt which guides sufficiently a lot of protein from the source of nature is not realistic.

[0009] Speaking of the synthetic producing method, the attempt which introduces the decode array of a gene into a bacteria expression vector, and discovers protein was not successful except for some cases. Bacteria production protein has weak solubility. The point of giving the result which a bacterial cell could not change eukaryote protein, and analysis of such protein mistook when modification after a translation was required about protein has another fault which uses a bacteria manifestation system. That is, generally bacteria production

protein has low solubility, and since there is a molecule deficit, usefulness has a limit.

[0010] It not only cannot produce enough a lot of protein, but by the usual laboratory-approach which makes a protein production object, the purity of production protein is not enough, either and variation is in purity. TrpE-RB fusion protein may be discovered for the example of representation of the difficulty at the time of producing a certain kind of protein production object, T7 RNA polymerase discovered to E.coli may be used for it, and a polypeptide may be produced. These approaches are comparatively complicated and need complicated elaborate biochemical actuation. Furthermore, since the target polypeptide does not carry out little deer production very much, these approaches have a big limit. Moreover, the polypeptide produced by this approach is seen in molecule, and it is common not to be desirable. For example, it is because this is not phosphoryl-ized.

[0011] Therefore, biochemical [desired] and the desired approach of producing a polypeptide with a biophysical property in a substantial amount are strongly desired from the importance of a gene production object polypeptide.

[0012] A remarkable advance is seen about an understanding of the function of the gene in living body health maintenance. If that it can generally say cannot produce protein with proper cytogene, various kinds of abnormalities will occur to a living body. It can ask that the whole gene is lost or the gene itself is missing by various kinds of causes for this reason that cannot be produced. From recognition of these factors, remarkable progress is accepted in a gene therapy.

[0013] For example, it is thought that the Wilms' tumor which is infantile kidney cancer is produced by gene inactivation of a chromosome 11. It is proved that oncogenicity can be controlled, if the normal chromosome 11 is introduced into a Wilms' tumor cell when the minute cell fusion medium transferring method of a single strand chromosome is used. On the other hand, this effectiveness is not accepted in Chromosome X and installation of 13.

[0014] On experiment level, although a certain extent of transition of a human chromosome without a deficit is useful, such transition is not applicable to the therapy of a genetic defect. Preparation of the suitable chromosome for a therapy not only needs remarkable skill, but requires time amount for one upwards, and it is because cost is high. For this reason, in many cases, this approach is nonpermissible.

[0015] When it is not desirable to use a chromosome without a deficit for a therapy, thinking theoretically below is introducing into a patient the proper whole gene or the part which has the effect at least. Although this approach has workability higher than the approach of introducing a chromosome without a deficit, it is only a certain fixed case that a gene therapy is desired.

[0016] Not only cost is very high, but separation of the proper nucleic-acid matter, sequencing, and cloning require time amount about this point. In addition, the complicated elaborate molecule genetic manipulation approach which can be used only in the location restricted very much also in the world is required of such an approach. Furthermore, such an approach produces a lot of matter suitable for a therapy even now.

[0017] As mentioned above, a specific therapy of the cell level which cost is comparatively low, and it is reliable, and can more generally use by using the bionics-approach, and uses the specific matter in a biochemical operation is desired strongly. Furthermore, a production object effective in a therapy is introduced on cell level, and a therapy which brings about change of neoplasm control etc. is also desired strongly. Although it is needless to say, it is strongly wished also about the production object which can produce so much in the state of purification without variation, and can be introduced into a deficit cell easily and effectively.

[0018] It is in the 1st purpose of this invention offering a specific therapy safe on the whole and and a production object effective in controlling cancer control.

[0019] It is in another purpose of this invention offering a cancer inhibitory-control production object and a therapy effective in the control and extermination using the bionics-approach and a production object of a cancer tumor. Moreover, it is also the purpose of this invention to offer the drugs constituent for cancer treatment which demonstrates an operation on cell level and the level between cells.

[0020] It is in still more nearly another purpose of this invention offering the drugs constituent which treats the symptom produced by the absence of the deficit or mutation antioncogene which makes nature or a synthetic production object an active ingredient, and an antioncogene.

[0021] This invention leads a specific antioncogene protein production object to an object cell, and consists of a cell therapy which realizes cancer control.

[0022] This invention offers the conventional radiotherapy and the cure for cancer with little need for a chemotherapy. Furthermore, this invention approach can be applied after discovery of disposition which is easy to

suffer from cancer hereditarily at the very early stage before tumorigenesis begins.

[0023] this invention — an antioncogene protein production object — good facilities — and in the point of using it at comparatively low cost and realizing cancer control on cell level, it is very advantageous.

[0024] Although there is a point which has not been clearly carried out about whether cancer will be generated enough when the antioncogene beyond one or it in a certain cell inactivates then, cell installation of a gene protein production object is new approach, and is the advantageous approach to a malignant tumor therapy. This invention is in the condition which suppressed the damage to a living body to the minimum unlike the cytotoxic cancer therapy from the former, and is still more advantageous in the point of bringing about an advantageous change on cell level.

[0025] As mentioned above, it is in the 1st purpose of this invention offering the approach of producing an activity gene production polypeptide without the deficit of a substantial amount.

[0026] Furthermore, another purpose of this invention has structure and a function in offering the same production approach of a specific gene production polypeptide as a natural student polypeptide.

[0027] If it summarizes, the above-mentioned purpose and other purposes of this invention are realizable by the approach of producing a substantial quantity of a request polypeptide by introducing a genetic material into an insect cell. For example, if a cloning gene, gene fragmentation, or its derivative is introduced into a host cell using a suitable vector, it is a substantial amount and the protein of a high grade can be produced with a high level.

[0028] This invention produces the polypeptide of high quality in a substantial amount, offers the approach of carrying out things of investigating a gene function on cell level, and is very advantageous.

[0029] Moreover, this invention is advantageous, is reliable, moreover, can produce this polypeptide at repetitive comparatively low cost, and is very advantageous also in this point.

[0030] Furthermore, this invention can produce the high quality polypeptide of a considerable amount which can solve an intergenic interaction on cell level and the level between cells, and is advantageous also at this point.

[0031]

[Easy explanation of a drawing]

Other purpose, descriptions, and these implementation approaches should become clear from the following publications at the above-mentioned above-mentioned purpose of this invention and the description, and the list. Moreover, it refers to an understanding of this invention itself at an accompanying drawing, and it should be obtained if the publication about the example of invention is read. Drawing 1 is the schematic diagram showing the configuration of the Baculoviridae manifestation vector which compounds pp110RB. Drawing 2 is the western blotting (Western blot) of a ppRB infection insect cell. Drawing 3 is western blotting which shows the cell extract extracted from the infected cell even 72 hours after infection. Drawing 4 is a microphotography in which the normal position between cells of RB protein is shown. Drawing 5 is the microphotography of infection Sf9 cell. Drawing 6 is a radioautograph which shows the result of phosphoryl-izing of RB protein in an insect cell, and dephosphorylation analysis. drawing 7 — pMG 3-245 — anti- — it is drawing showing the rough lysate from -RB, infection Sf9 cell, and the electrophoresis analysis result of eluate. Drawing 8 shows the fusion protein and the south Western (Southwestern) DNA binding measuring method of Baculoviridae-manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10%. It is the radioautograph of the blot from parallel gel to the gel used in order that drawing 9 might create drawing 8, and the incubation of this blot is carried out by the 32P-labeling DNA fragment. It is the chromatogram which shows complexing of drawing 10 Baculoviridae-manifestation RB protein and an SV40 T antigen. The nuclear transformation of the purification RB protein after the microinjection to the cytoplasm of drawing 11 Saos-2 cell is shown.

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EXAMPLE

[The best example of invention] All have adopted the contents here and the reference which makes reference on these specifications constitutes a part of this specification publication. Hereafter, this invention will be explained in the following order.

A. an outline -- the nuclear transformation B7. epitome [0033] of specific complex formation B6. purification RB protein with the RB protein purification B5.DNA-avidity from the phosphoryl-ized B4. infection insect cell after the nuclear normal position of the manifestation B3. extraversion RB protein of the extraversion RB protein in the configuration B-2. infection insect cell of example of B.RB gene production object B1. recombination Baculoviridae, and a translation, and an SV40 T antigen A. When outline this invention introduces a genetic material into an insect cell culture object, it can be used for this gene protein producing a specific gene protein production object by culture about the approach of producing gene protein. If this approach is used, the deficit with a high force value refined substantially can twist, and activity protein can be obtained biochemically.

[0034] The manifestation system of this invention has a large application. That is, for example, Homo sapiens, the cloning gene of an animal, its fragmentation, a homologue, a derivative, or a part is applicable to production of a request protein production object. Thus, needless to say, the produced protein is applicable to the therapy of a deficit cell at the elucidation of a gene function. a flume -- obtaining -- it is because a gene interacts on cell level.

[0035] Since the insect cell culture object had an eukaryote property when a gene protein production object was produced, the suitable thing was found out. In order to introduce a genetic material into a cell culture object, the usual vectors, such as a virus vector, can be used.

[0036] For example, in order to produce RB gene protein production object, the virus vector of an insect cell culture object is used. In order to rearrange with a high level in a culture insect cell and to produce protein about this point, Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.

[0037] This invention is a cell therapy which introduces a specific antioncogene protein production object into an object cell, and realizes neoplasm control. Since a given cell has a deficit gene, in the point which causes the protein manifestation deficit in a cell, it may be missing.

[0038] It is related with this invention approach using the gene protein production object related to a deficit gene. A purification protein production object is introduced into an object cell, for example, neoplasm control is realized. By supporting and introducing this production object into support suitable in pharmacology, this protein production object is cell level, or acts on the bottom level of a cell.

[0039] the cell RB gene protein which whose RB gene which is an antioncogene is missing as an example of this approach, or does not have it was boiled and introduced. What is necessary is just to refer to the parent-patent application specification which has made reference on these specifications about the detailed information about RB gene.

[0040] In the suitable example, the small-child cancer which is a retinoblastoma, i.e., a symptom with the rare

retina under growth, as a PM of recessive tumorigenesis research is used. Based on the normal position and its recessive nature of the heredity element to a chromosome 13q14 which is involving, the gene presumed to be an antioncogene, i.e., a retinoblastoma susceptibility gene, (RB) was cloned. A 4.7 kilobase mRNA imprint is discovered at all in the normal organization which the exon of 27 distributed this gene to the genomic DNA of 200 kilobases, and inspected. By the sequence analysis of a complementary DNA clone, the long open reading frame which has coded the hypothetical protein of the amino acid of 928 became clear. When the antibody to the predetermined epitope guessed from the RBcDNA array was used, as for this RB gene production object, relative molecular weight (Mr) was identified the nucleus phosphoprotein of 110,000-114,000. This was named pp110RB. [0041] only to a retinoblastoma, it has become clear that the deficit of RB gene function is boiled also at the neoplasm of some of others including a breast cancer, an osteosarcoma, a prostatic cancer, or small cell lung cancer again, and is involving. According to the latest research, if RB gene is reintroduced into a retinoblastoma, an osteosarcoma, or a prostate gland cancer cell by retrovirus medium gene transition, it will have become clear that some fields of new phenotypes including the tumorigenesis in a nude mouse are controlled clearly. This is a direct proof of the neoplasm control function of RB gene. However, the foundation in the molecular level of this biological activity is not established yet.

[0042] Although all the biochemical properties and biological functions of an antioncogene production object are not solved the place to current, it is because there is little this protein in a cell as for this, so it is difficult to obtain in sufficient amount. What is necessary is just to refer to the parent application specification (the name of invention: the production approach of a gene protein production object) for which it applied to this application and coincidence about this point.

[0043] The cell therapy which has a deficit gene or introduces a specific gene protein production object into a cell without a gene was invented. According to this invention, the deficit refined substantially twists and the proper amount of activity gene production protein can be biochemically introduced into a deficit gene with an effective dose on a therapy.

[0044] The cell therapy of this invention has a large application application. This protein production object is useful not only to the therapy of a deficit cell but the elucidation of a gene function. It is because a gene interacts on cell level.

[0045] In the one specific example, a retinoblastoma gene protein production object, i.e., pp110RB, has a deficit gene, or it can apply it to the therapy of an eukaryote cell without a gene widely.

[0046] In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed the SV40 T antigen and the specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0047] B. Explain in more detail about the example of RB gene production, next the production approach by this invention of pp110RB protein. What is necessary is just to refer to the following parent-patent application specifications about information detailed to the pan about this protein and a retinoblastoma gene.

[0048] Although all the biochemical property and biological functions of an antioncogene production object, such as RB gene, are not solved, this is because it is difficult to obtain purification protein in sufficient amount. That is, it is because it is because a cell has little protein and the protein manifestation by the installation to the bacteria expression vector of the coding array of a gene has not succeeded in one except for a part in addition again. It has been concluded that the problem which the approach used now has from these was avoidable if a cloning gene is discovered in an eukaryote system. Although it will be an antioncogene if this invention is illustrated, although the specific example of this invention is related with production of RB gene protein production object, it can apply also to production of the protein production object of other eukaryote genes which are not restricted to this, therefore is related also with production of such a protein production object.

[0049] In order to produce recombination protein with a high level in a culture insect cell, it is desirable although Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced

includes the same modification after a translation as the protein looked at by the original high-class eukaryote.

[0050] In order to test the manifestation possibility of functional RB protein by the Baculoviridae system, cloning Homo sapiens RBcDNA including the perfect coding array of RB stirp was introduced into the AcNPV expression vector, and the recombination virus was bred in the insect cell. The Homo sapiens pp110RB manifestation was realizable with a high level with the host-vector system. The obtained protein was phosphoryl-ized and the nucleus of an infected cell was correctly made into the target. Furthermore, the purification method of RB protein was also established. In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed an SV40 T antigen and specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0051] In order to produce RB protein in the configuration Baculoviridae manifestation system of B1. recombination Baculoviridae to the maximum extent, it rearranged by removing a great portion of 5' non-coding array from RB gene, and the transition vector was constituted. By seat singularity mutagenesis, two BamH1 seats were introduced into NUKURECHIODO 116 and 2935 of RBcDNA, and the configuration of a recombination transition vector was promoted. PORIHE drine compounds 5' a non-coding array with refined pAcYM1/RB2.8 [perfect (60 base pairs) as shown in drawing 1] — 5' of RBcDNA — it unites with a non-translated field and mRAN which a perfect coding array follows after that is coded. This recombination gene does not contain an ATG codon for the upstream of NUKURECHIODO 139 of Shinsei RB initiation seat. Therefore, a recombination gene codes un-uniting and full length RB protein.

[0052] Although the transition vector pAcYM1 is shown in drawing 1 , these all have the upper array of the PORIHE drine compounds gene containing A of an initiation ATG codon, and BamH1 seat characteristic next continues. There is a publication of Matsuura etc. about a transition vector. J. Gen.Virol., 68:1233-1250 (1987). pRB 44-2 includes the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning in BamH1 seat of the plasma pGEM1 (Promega). About the recombination Baculoviridae vector, pAcYM1/RB2.8 [i.e.,], since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed in the bottom of direct control of a polyhedrin promotor.

[0053] The following points were taken into consideration, when a Baculoviridae manifestation vector was constituted and pp110RB was compounded. pRB 44-2 becomes BamH1 seat of pGEM1 from the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning. Including EcoR1 fragmentation of about 7 kbs of a virus DNA array located in the side face of a PORIHE drine compounds gene, in this case, pAcYM1 permutes all the PORIHE drine compounds coding arrays except the 1st A of ATG by BamH1 linker, although the leader sequence remains as it is. Since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed about a recombination Baculoviridae vector including polyhedrin promotor RBcDNA fusion, pAcYM1/RB2.8 [i.e.,], in the bottom of direct control of a polyhedrin promotor. The array of a fusion joint is shown in the direction under drawing 1 . Moreover, the case notation under drawing 1 shows a polyhedrin promotor, and the upper case notation shows a RBcDNA array. And BamH1 linker is underlined. An arrow head shows the translation of the fusion gene using ATG (nucleotide 139) of RB. a* (+1) of drawing 1 shows the 1st A of the translation initiation codon ATG of a PORIHE drine compounds gene.

[0054] The imprint to a viral genome from the recombination plasmid of RBcDNA is a wild type Autographa by the RIPOFE cushion (lipofection). It carried out by carrying out the conte run SUFE cushion (contransfection) of the PaCym1/RB2.8DNA using a californica nuclear polyhedrosis virus DNA (BRL). Since a PORIHE drine compounds closing object was not accepted in an infected cell, the recombination virus which inactivated the PORIHE drine compounds gene by the ARERIKKU permutation with RB gene by family recombination was identified according to the plaque gestalt. Plaque purification of the virus was carried out 3 times, and the pure Baculoviridae stock was obtained. This was set to AcNPV-Y4RB.

[0055] Before the manifestation AcNPV polyhedrin promotor of the exogenous RB protein in B-2. infection insect cell determines whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell Sf9 cell is prepared. The yeast rate of 3.33 gm/l (yeastolate), insect culture-medium (Grace's insect medium)-Bull.1555 (a Texas (1987) agricultural experiment place —) of the grace which added a lactalbumin hydrolysis production object (GIBCO) and 10% of heating inactivation fetal calf serum (GIMINI), A college experiment place and TX are used and it is Sf9, i.e., Spodoptera, by the monolayer or suspension culture at 27 degrees C. frugiperda

IPLB-Sf21-AE [In vitro, 13:213-217 (1977)] was cultivated. When carrying out large-scale production of the cell lysate, the spinner culture of Sf9 cell was cultivated by the EX-CELL400 convention culture medium (J. R.Scientific) which does not contain a blood serum. Suspension culture of the malt (Molt) -4 cell of Homo sapiens T leukemia cell lineage was carried out by RPMI1640 culture medium which added the calf serum 20%. Saos-2 cell of osteosarcoma cell lineage was cultivated by the Dulbecco (Dulbecco) denaturation Eagle's medium which added fetal calf serum 7.5%.

[0056] When an AcNPV PORIHE drine compounds prow motor determined whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell, infection processing of the Sf9 cell was carried out by plaque purification AcNPV-Y4RB. 40 hours after infection, the lysate of an infected cell was collected and immunoprecipitation processing was carried out with anti-RBO-47 antigen. Next, after carrying out SDS-PAGE processing of the sample, western blotting analysis was carried out.

[0057] The pp110RB identification result in the AcNPV-Y4RB infection insect cell performed to drawing 2 and drawing 3 by western blotting analysis is shown. In the case of drawing 2, the cell extract was prepared 40 hours after infection from the simulation infected cell (path 2), the AcNPV-Y4RB infected cell (path 3), or wild type AcNPV infection Sf9 cell (path 4). The malt -4 of Homo sapiens leukemia cell lineage was used as contrast (path 1).

[0058] Although the manifestation was accepted in the extract (path 3) of an AcNPV-Y4RB infected cell in RB protein of the same full length as a mammalian cell (path 1) when immunity blot processing was carried out by the monoclonal system antibody as shown in drawing 3, in the case of the simulation infected cell or the wild type AcNPV infected cell (paths 2 and 4), it did not accept. In order to ask for the optimal stage of RB protein production, in the case of drawing 3, the time amount after infection was changed, and the cell extract was obtained from AcNPV-Y4 infected cell in it. Immunoprecipitation processing of the lysate was carried out with anti-RBO.47 antigen, and immunity blot processing was carried out by the pMG3-245 monoclonal system antibody. p110RB of drawing 3 and pp110RB express non-phosphoryl-ized protein and phosphoryl-ized protein, respectively. After infection acted as the monitor of the production of RB protein, and asked for the optimal stage to collect cells. As shown in drawing 3, RB protein production could be detected 24 hours after infection, and the 12 next hours increased the amount of production considerably. Protein production level was maintained all the time over about 72 hours after infection, and the bacteriolysis of a cell started it substantially 72 hours after. In order to suppress protein degradation accompanying lysis to the minimum, infected cells were usually collected about 40 hours after infection.

[0059] When detecting the manifestation of RB protein, AcNPV-Y4RB was used and infection processing of the Sf9 cell was carried out by MOI of 0.5. The cell of 5x10⁴ was dissolved 24, 36, 48, 60, and 72 hours after from infection in the 1ml dissolution buffer solution (Naf [of Nonidet (Nonidet) P-40;1mM / of EDTA;100mM] of NaCl;50mM of NaCl of 50mM, and 7.4; 0.2% of pH, and PMSF of 1mM), respectively, at-long-intervals alignment processing was carried out for 5 minutes, and the lysate was defecated. Next, the incubation of the lysate was carried out with anti-RBO.47 antigen, and the immune precipitate was separated by SDS-PAGE 7.5%. Then, protein was moved to nitrocellulose paper and it processed with the conventional method. After leaving it in an overnight cut off state and carrying out incubation processing of the nitrocellulose paper with pMG3-245 anti-rRB monoclonal system antigen for 3 hours, according to Cell and the publication of 54:275-283 (1988), it processed by the alkaline phosphatase junction goat **-mouse IgG and the chromophoric substrate.

[0060] As for the phosphoryl-ized RB gene after the nuclear normal position of B3. exogenous RB protein, and a translation, molecular weight (Mr) codes the nucleus phosphoprotein of 110,000. In order to investigate whether RB protein produced into the insect cell by Baculoviridae reached the nucleus correctly, 40 hours after infection, anti-RBO.47 antigen was used and immunity dyeing of the AcNPV-Y4RB-infection Sf9 cell was carried out. The normal position between cells of RB protein discovered into the insect cell by immunity dyeing is shown in drawing 4 and drawing 5. The case of AcNPV-Y4RB-infection Sf9 cell is shown in the case of mock infection Sf9 cell, and drawing 5 at drawing 4. As shown in drawing 4 and drawing 5, the infected cell contained the unusually large nucleus. Such a condition is peculiar to the cytopathogenic effect of the Baculoviridae infection. Dyeing was not accepted when the incubation of mock infection or the wild type AcNPV infection Sf9 cell was carried out with anti-RBO.47 antigen (drawing 4). However, dyeing strong only against the nucleus of an AcNPV-Y4RB infected cell was accepted (drawing 5). When SDS-PAGE and Western blotting analyzed the nucleus and cytoplasm extract from AcNPV-Y4RB infection Sf9 cell, it has checked that exogenous RB protein mainly existed in a nucleus part.

[0061] Immunity dyeing was performed at the following process. Also in both mock infection wild type AcNPV infection and AcNPV-Y4 infection the overnight incubation of the Sf9 cell was inoculated and carried out to the chamber slide (Miles Scientific) which carried out coating of the Polly L-lysine (Sigma) 40 hours after infection. Before moving from all to the following process, the phosphoric-acid buffer sodium chloride water solution washed the slide. That is, the interstitial cell was fixed for 20 minutes for 10 minutes using the acetone (-20 degrees C) using the 0.04M phosphate buffer solution of 4% formaldehyde of ****. After carrying out the pre-incubation of the fixed cell for 10 minutes with the PBS solution of 2% of normal goat blood serum, the overnight incubation was carried out with anti-RB0.47 antigen diluted to 0.02% of triton X-100. The BIOCHINIRU-ized goat **-rabbit IgG (Canada and TAGO of Burlingame) was added after washing. 1 hour after, after carrying out the incubation of the interstitial cell for 45 minutes using AB complex joined by the horseradish peroxidase (Canada and Vector Labs of Burlingame), the incubation was carried out using the following substrate. This substrate consisted of 0.05% of 3 and 3'-diaminobenzidine tetrahydro chloride, and 0.01% of a 0.05M tris-HCl solution of H₂O₂. pH7.6 (Sigma). The reaction was suspended by carrying out PBS washing of the cell after 3 - 5 minutes. Next, a photograph of a cell was taken with the NIKON diamond photograph microscope (diaphotomicroscope).

[0062] The result of phosphoryl-izing of RB protein produced into the insect cell at drawing 6 and dephosphorylation is shown. 40 hours after AcNPV-Y4RB infection, a 35S-methionine or 32P-orthophosphoric acid was used, and metabolic turnover labeling of the Sf9 cell was carried out in 3 hours. Malt -4 was used as contrast and immunoprecipitation of the cell lysate was carried out using anti-RB0.47 antigen. before carrying out potato acid phosphatase (PAP) processing, (paths 1, 2, 3, and 4) were separated, SDS-PAGE (path 1', 2', 3', and 4 -- ') separated 35S- and a 32P-labeling RB protein immune complex after processing, and radioautography analyzed. Similarly, the dephosphorylation experiment was conducted using the lysate from a non-labeled cell, and western blotting analysis was performed before and after potato acid phosphatase processing (paths 5 and 6 and 5', 6').

[0063] Furthermore, the case of drawing 4 is explained continuously. Phosphoryl-ization of RB protein is produced in a multiplex serine and threonine residue, and the molecular weight heterogeneity of RB protein in SDS-PAGE is explained. In order to investigate whether phosphoryl-ization is received after RB protein produced into [Oncogene Res., 1:205-214(1989);Cell, 56:57-65 (1989)] insect cell translating, a 35S-methionine or 32P-orthophosphoric acid was used from 3 hours after infection in 40 hours, and metabolic turnover labeling of the AcNPV-Y4RB-infection Sf9 cell was carried out. Immunoprecipitation processing of the cell extract was carried out, and radioautography processing was carried out after carrying out SDS-PAGE analysis. What is necessary is just to refer to the paths 2 and 4 of drawing 6 about this point, respectively. It was parallel to this, the immunoprecipitation nature RB protein from the same extract was processed by potato acid phosphatase (PAP), and the dephosphorylation effectiveness RB protein migratory [in SDS-PAGE] was tested. The molecular weight 110,000 of 35S-labeling RB protein fell from the double band to 1-fold band after dephosphorylation (path 2' of drawing 6). And activity emitted almost completely from 32P-labeling RB protein (path 4' of drawing 6). The band change pattern as PAP processing also with the same dephosphorylation analysis by Western blotting about the lysate from a non-labeled cell which carried out infection processing by AcNPV-Y4RB was shown (the path 6 of drawing 6 , 6'). These observation result showed having phosphoryl-ized RB protein produced into the insect cell. Moreover, the molecular weight heterogeneity of RB protein accepted by SDS-PAGE from this change has been explained.

[0064] Radiation-labeling and dephosphorylation analysis of Sf9 insect cell were carried out at the process shown below. 40 hours after infection, the incubation of the Sf9 during 30 minutes cell (3x10⁶) was carried out using the 60mm pan by the methionine and the DME culture medium containing neither of a phosphoric acid which added 10% of fetal calf serum. Then, metabolic turnover labeling of the cell was carried out by adding a 0.25 mCi(s)/a ml 35S-methionine (1134 Ci/mmole, NEN) or 0.25 mCi(s)/ml 32P-orthophosphoric acid (support nothing, ICN) in 3 hours. The cell was extracted using the dissolution buffer solution (tris of 50mM - NaF [of Nonidet P-40;1mM / of EDTA;100mM] of NaCl;50mM of HCl and 7.4; 0.2% of pH, and PMSF of 1mM), and immunoprecipitation processing of the extract was carried out with anti-RB0.47 antigen.

[0065] Potato acid phosphatase (PAP and Boehringer) dephosphorylation analysis of two thirds of the immunoprecipitation RB protein from 35S- or the 32P-labeling cell lysate, and the non-labeled cell lysate was carried out. [Oncogene Res., 1:205-214 (1989)]. The incubation of the RB protein content immune complex was carried out using PAP of 1.5 units in 37 degrees C (leupeptin of MES of 20mM, and 2; 50micro [of NaCl;1MgCl(s) of mM of pH5.5;100mM] M) of reaction buffer solutions, and 60 minutes, and after the reaction, after carrying out SDS-PAGE analysis 7.5%, radioautography or Western blotting analyzed RB protein.

[0066] Infection processing of the Sf9 cell was carried out by AcNPV-Y4RB by the multiplicity of infection (MOI) of purification 1.0 of RB protein from B4. infection insect cell, and the cell lysate was obtained 40 hours after infection. The total level of RB protein discovered in the Baculoviridae system on this condition was about 17-18mg per 1l. of infection insect cell culture objects ("109 cell). About this point, **** which illuminates Table 1 3 is good.

[0067]

[Table 1]

Table [] 1 purification process of the recombination RB protein from the Baculoviridae infection insect cell Sum total tongue RB tongue Yield Whenever [purification] Purity Park mass Park mass (%) (%)
(mg) (mg) A cell extract 670a 16c 90c 1.0 times 2.3pMG3-245 immunity affinity column 13.5b 12.9d 72 cds 41.3 times 95 Protein quantum c. western blotting by the protein quantum b. micro BCA (PIERCE) by the approach and spectrophotometry of a. Bradford (Bradford) (Bio-RAD) And the protein quantum by the protein quantum d. Coomassie-brilliant-blue-staining method by the density measurement method, and the density measurement method [0068] As shown in Table 1, 90% of discovered RB protein (16mg) was accepted in the supernatant after cell division, and 10% had stopped at the insoluble fraction. Since 2.3% of all cell protein was formed, RB protein was easily detectable from the cell lysate. About 135mg protein was recoverable from the alkaline eluate of a column after one-step immunity affinity chromatography purification. In order to investigate the purity of elution RB protein, SDS-PAGE and Coomassie brilliant blue staining analyzed the eluate aliquot equivalent to 2.5×10^5 cell.

[0069] What is necessary is just to refer to drawing 7 which shows immunity affinity chromatography purification of pp110RB about this point. After analyzing the aliquot (equivalent to 2.5×10^5 infected cell) of eluate with the electrophoresis method by 10%SDS-poly AKURIRO amide gel from the rough lysate from mock infection Sf9 cell (path 1) or AcNPV-Y4RB infection Sf9 cell (path 2) of 1×10^5 , and pMG3-245 anti-RB immunity affinity chromatography, it analyzed by the Coomassie-brilliant-blue-staining method. The arrow head has shown RB protein of anticipation molecular weight.

[0070] When judged by the density measurement method, the above-mentioned one-step purification process is effective, and RB protein was able to be obtained by 41.3 times whenever [95% / of purity / (drawing 7 , path 3), 72% / of yield / , and purification] (Table 1).

[0071] Some modification was added although the immunity affinity column was constituted according to the publication of Shneider, etc. ****, etc. [J. Biol.Chem., 257:10766-10769(1982);Virology, 144:88-100(1985)]. After filling up the Bio-Rad (Bio-Rad) column with 2ml protein G-agarose (Genex) and washing by 0.01-N HCl, the joint buffer solution (sodium acetate of 0.1M, NaCl of pH5.0;0.1M) washed. It joined together by adding the anti-rRB monoclonal antigen (pMG 3-245) of 2mg [15] to a column. Next, the boric acid buffer solution of 0.1M washed the column widely, and the bead was re-suspended in the 20ml buffer solution. Dimethyl PIMERIMI date dihydrochloride (Sigma) was added so that the last concentration might be set to 40mM(s), mixture was stirred at the room temperature for 1 hour, and crosslinking reaction was performed. after washing and a room temperature -- for 10 minutes, 20ml solution of ethanolamine-0.1M boric acid buffer solutions of HCl of 40mM, and pH8.0 -- it came out and the residual reactivity radical of a bead was intercepted. then, the glycine of 0.2M and pH2.3 -- it came out and the column was washed, and it saved in this solution until it neutralized and was needed with tris buffers (tris-HCl of 50mM, EDTA [of pH7.4;100mM] of NaCl;1mM of PMSF:1mM). When OD280 of the first monoclonal antigen sample and OD280 of the flow fraction in the following process were measured, about 10mg pMG 3-245 had combined with the protein G-agarose which is 2ml.

[0072] About RB protein, two biochemical properties are described despite specific complex formation current [with B5.DNA avidity and an SV40 T antigen]. That is, it is one of them that DNA is combinable as a peculiar property, and it is one more [Nature, 329:642-645 (1987)] and that how many kinds of the neoplasm protein and the specific complex of that DNA tumor virus can be formed [Cell, 54:275-283,(1988) Science, 243:934-937 (1989);Nature (London), 334:124-129 (1988)]. About these [which are participating in proteinic biological functions] two well-known biochemical properties, RB protein refined from the Baculoviridae infection insect cell was tested.

[0073] A south Western DNA-joint measuring method is shown in drawing 8 and drawing 9 . The purification trpE-RB fusion protein of 6microg and purification Baculoviridae manifestation pp110RB were applied to SDS-PAGE 10%. In the case of the joint measuring method shown in drawing 8 , the Coomassie-brilliant-blue-staining method was used, and, in the case of the joint measuring method shown in drawing 9 , the electric imprint of the parallel

gel was carried out at nitrocellulose paper. Next, the incubation of the blot was carried out by the ^{32}P -labeling DNA fraction, and radioautography analysis was carried out. In the case of drawing 8 and drawing 9, it is as follows. Path 1:RB19-22; path: — RB23-27; — path 3:RB19 — the purification RB protein from a -27; path 4:AcNPV-Y4RB infection insect cell.

[0074] In the case of drawing 8 and drawing 9, DNA binding was measured by south Western analysis. In this case, SDS-PAGE separated the trpE-RB fusion protein of tales doses, and the purification RB protein from an insect cell 10%. The amount of the protein by which loading was carried out was calculated by the Coomassie-brilliant-blue-staining method (drawing 8). In the experiment which used another parallel gel, after carrying out the electric imprint, the incubation was carried out to the nitrocellulose membrane by the ^{32}P -labeling DNA. Radioautography analyzed next DNA combined with protein (drawing 9). The affinity to DNA of the fusion protein RB 19-27 with which the main domain interacts with DNA is 20 times as high as two fields RB 19-22 and RB 23-27. About this point, the path 3 of drawing 9 could be compared with paths 1 and 2, and purification full length RB protein showed strong DNA avidity like RB 19-27 (drawing 9 , path 4). The DNA avidity of the purification RB protein from an insect cell is elution which continues after that from a column even if based on maintenance of the protein by the DNA cellulose. — It has proved also by NaCl- of about 400 mM(s).

[0075] Purification of pp100RB from an infection insect cell was carried out as follows. By MOI of 1.0, using AcNPV-Y4RB, infection processing was carried out and suspension culture of the Sf9 cell was carried out (1X166 cell / ml, 1000ml). 40 hours after infection, low-speed centrifugal separation pelletizes and washes a cell, and it is tris of 50mM(s). — It re-suspended in the extract buffer solution containing NaCl;10% the leupeptin of PMSF;25microg of DTT;1mM and 50 units of NP-40;1mM of glycerol (v/v);1mM of HCl and 7.4; 0.2% of pH / aprotinin of ml. [of EDTA;100mM] After carrying out an incubation for 15 minutes in Hikami, centrifugal (for 10,000xg, 4-degree-C, and 10 minutes) clarification processing of the sample was carried out, and RB content supernatants were collected. As mentioned above, immunity affinity chromatography of pp110RB was carried out in the column of 2ml capacity containing the anti-fRB monoclonal antigen (pMG 3-245) combined with protein G-agarose. After letting a supernatant pass 4 times in a column, the following solution was used in the amount of 200 bed capacity, respectively, and the column was washed continuously. The dissolution buffer solution, the dissolution buffer solution containing NaCl of 500mM, and a penetrant remover (PMSF;10% glycerol [of 200mM / of NaCl;1mM] of EDTA;1mM of DTT;1mM). Next, binding protein was eluted from the column with the alkaline elution buffer solution containing PMSF of EDTA;1mM and 10% of glycerol of NaCl;1mM of the triethylamine of 2mM, and pH10.8;200mM. 1ml fractions are collected and it is tris of 1M immediately. — It neutralized by 1/12 capacity of HCl (pH7.5), and saved at -70 degrees C in 10% glycerol.

[0076] When refining pp110RB from an infection insect cell, after calculating the total amount of protein, the south Western DNA binding measuring method and the SV40 T antigen joint measuring method were enforced.

[0077] The amount of all the protein of the elution fraction of an immunity affinity column was calculated with the micro-BCA measuring method (PIERCE). SDS-PAGE analyzed the elution protein sample next, and the Coomassie-brilliant-blue-staining method and the degree were asked for the amount of RB protein of eluate by the density measurement method. The total amount of protein of a cell extract was measured by the approach (Bio-Rad) of Bradford. [Anal.Biochem., 72:248-254 (1976)]. In order to carry out the quantum of the RB protein of the cell lysate, after using the purification RB protein diluted in order as a criterion and carrying out Western blotting, density measurement compared band strength. What is necessary is just to refer to Table 1 about this point.

[0078] Protein blotting was carried out with the conventional method. According to Bowen's (Bowen) etc. publication, the incubation of the blot was carried out by the radiation labeling DNA. [Nucleic Acids Res.8:1-21 (1980)]. This was performed at the room temperature. After rinsing the blot quickly, it washed 3 times by the urea of 6M, and 0.2% of NP-40 (all are 20 minutes), and washing (all are 30 minutes) was succeedingly carried out 4 times with the DNA binding buffer solution (NaCl;0.2% BSA;0.2% Ficoll400 and 0.2% of polyvinyl pyrrolone of EDTA;50mM of tris-HCl of 10mM, and pH7.0;1mM). Next, the incubation of the blot was carried out for 30 minutes in the DNA binding buffer solution including the ^{32}P -labeling DNA. By EcoR1, line-ized pGEM1DNA was labeled by the alpha- ^{32}P deoxy nucleotide (Amersham, >3000 Ci/mmol) by the random priming, and it was used as a probe. Washing of the blot was carried out 3 times (all are 10 minutes) with the DNA binding buffer solution after hybridization, it was air-dry and radioautography analyzed. TrpE-RB fusion protein was included as contrast. Each trpE-RB fusion protein was named according to the exon of RB gene which protein contains. That is, RB 19-22, RB 23-27, and RB 19-27 occupy the pp110RB field of an exon 19-22 (amino acid 612-775), an exon 23-27 (amino

acid 776-928), and an exon 19-27 (amino acid 612-928), respectively.

[0079] Immunity chromatography purification of the SV40 T antigen was carried out from Ad-SVX1-infection 293 cell. [J. Virol., 53:1001-1004(1985); Cold Spring Harbor Press., Cold Spring Harbor, NY, pp.187-192(1982)]. And anti-T monoclonal PAG419 antigen was obtained from Oncogene. The well-known complex formation measuring method was enforced. However, some modification was added. That is, the Baculoviridae manifestation RB protein of 800ng was mixed with the 1ml EBD buffer solution (NaCl of tris-HCl of 50mM, and pH8.0; 120mM, and 0.5% of Nonidet P-40) containing PMSF of 1mM, 25microg [/ml] leupeptin and 50 units / aprotinin of ml. The purification T of 800ng(s) was added to mixture, and the incubation was carried out in Hikami during 90 minutes. anti- — using either of -RB0.47 or PAB419 antigens, immunoprecipitation of the aliquot of mixture was carried out and Western-blotting analysis was carried out. The blot made it react with pMG 3-245 and PAB419 in order. After carrying out an incubation with the alkaline phosphatase junction goat **—mouse IgG, coloring processing of the blot was carried out using the color-enhancing substrate.

[0080] In order to test the capacity for purification RB protein to be able to form an SV40 T antigen and specific complex, equivalent RB protein and an equivalent T antigen were mixed, and immunoprecipitation of the aliquot of mixture was carried out using anti—RB0.47 antibody or -anti-T antigen PAB419.

[0081] In relation to this point, the complex which Baculoviridae manifestation RB protein and an SV40 T antigen form in drawing 10 is shown. Purification Baculoviridae manifestation RB protein was mixed with the purification T antigen by in vitro one. next, the mixture of this aliquot — PAB419 (path 2) — anti- — immunoprecipitation was carried out by either of -RB(s)0.47 (path 3), and Western blotting analyzed. Paths 1 and 4 show the purification Baculoviridae manifestation RB protein which carried out immunoprecipitation by the purification SV40 T antigen which carried out immunoprecipitation by PAB419, respectively, and anti—RB0.47 antibody.

[0082] As shown in drawing 10 , when the T antigen was mixed with RB protein by in vitro one, PAB419 not only coimmunoprecipitates with RB protein, but (the path 2), and T and anti—RB0.47 antigen coimmunoprecipitated (path 3). It is proved [data / these] that RB protein from the Baculoviridae infection insect cell can form an SV40 T antigen and specific complex.

[0083] Since it became clear that the nuclear transformation purification protein of B6. purification RB protein had the two well-known biochemical properties of RB by in vitro one, it investigated about the in vitro behavior of purification protein next. The cytoplasm of Saos-2 cell with the osteosarcoma cell lineage without an exon 21-27 which codes C terminal cutting RB protein (p95), including a deficit RB gene was injected with purification RB protein. [Proc.Natl.Acad.Sci.U.S.A., 87:6-10 (1990)]. The antibody orientated this protein to the cytoplasm in consideration of the fact of going to the C terminal of RB protein, in the small quantity of extent which is not accepted by anti—RB0.47 antibody to be used. Immediately after injection, the cell was fixed and it analyzed by the immunity staining technique. The nuclear normal position of generation RB protein after carrying out a microinjection to drawing 11 to the cytoplasm of Saos-2 cell is shown. The cell was injected with generation RB protein and it analyzed by the immunity staining technique. The arrow head shows among drawing that dyeing of the nucleus after a microinjection is strong compared with the non-injecting cell.

[0084] As shown in drawing 11 , compared with a non-injecting cell, dyeing of the nucleus after a microinjection is strong (arrow head), and it is shown that injection protein was immediately conveyed to the nucleus. since RB protein is known as a nucleoprotein — the nuclear normal position of after a microinjection and purification protein — immediately — and although cut correctly, this has suggested that this protein is activity in in vivo one.

[0085] In the case of the microinjection, purification RB protein was dialyzed so that the last concentration might become [ml] the injection buffer solution containing DTT of EDTA; 0.1mM and 2% of glycerol of KCl; 0.1mM of tris-HCl of 20mM, and pH7.4; 10mM in 0.5mg /. The capillary glass tube needle (Eppendorf) was used and the microinjection of the Saos-2 cell which grew on the glass CHANN bar slide was carried out by the usual approach. The micro manipulator and opposition contrast microscope (NIKON make) of Eppendorf which attached the vacuum pressurizer were used, and micromanipulation of the capillary tube was carried out, respectively, and the microinjection process was visualized. After the microinjection, the cell was immediately fixed with the 0.04M phosphate buffer solution solution (pH7.4) of formaldehyde 4%, and it analyzed by the immunity staining technique.

[0086] As explained more than B6. epitome, it has proved that the bottom Homo sapiens retinoblastoma gene production object of the transcriptional control of the Baculoviridae polyhedrin promotor could be efficiently discovered. Since RB protein had the misgiving which it not only blocks growth of a cell, but shows "toxicity" to it depending on the case, it was thought that the attempt which has discovered RB protein on high level for a long time was difficult. The imprint of the foreign gene from a polyhedrin promotor is produced in the second half of

infection, and cutoff of production of an extracellular virus particle, a cell, and a great portion of virogene continues next. Therefore, when superfluous production is carried out, the Baculoviridae insect cell lineage is advantageous to composition of protein, such as RB protein which has that it is harmful to cell growth. Another advantage of this system is in the similarity of an insect and the protein synthesis path of a mammalian cell. [0087] Moreover, it also becomes clear that RB protein reaches the nucleus of an insect cell correctly, and this connotes that the nuclear normal position signal of mammalian is recognized by the insect cell. Although glycosylation of the recombination protein in the Baculoviridae manifestation system is restricted to 0-association of a high mannose mold, and an N-joint oligosaccharide, suitable phosphoryl-ization of outpatient department protein is reported about c-myc and the manifestation of HTLV-Ip40x (J. Virol.). Although RB protein was phosphoryl-ized from before, not carrying out glycosylation was known. This is a reason for production of functional RB protein with the suitable Baculoviridae manifestation system.

[0088] As indicated on these specifications, RB protein produced into the infection insect cell is phosphoryl-ized after a translation, therefore can specialize a multiplex band by Western-blotting analysis completely like the case of RB protein of Shinsei mammalian. However, when seeing from band strength and it compares with hyper-phosphoryl-ized RB protein, phosphoryl[non-]-izing and a phosphoryl-ized gestalt are superior. Since this phenomenon's being reflection of the cell cycle condition of the population at the time of viral lytic infection or a lot of exogenouses RB exist in a cell the place to current, it is not known whether it is because phosphoryl-izing of the protein by the insect kinase is only inadequate. In order to determine the same thing **** as the case of phosphoryl-ized PATANGA mammalian protein, and accuracy, it is necessary to map the phosphoryl-ized location of RB protein in a precision.

[0089] The total level of the recombination RB protein discovered in the Baculoviridae system is about 17 to 18 mg per 1l. (109 cells) of infection insect cell culture objects. This manifestation level is equivalent to other mammalian protein which this system generates. For example, in the case of interleukin 2, it is 10-20 mg/l [The Banbury Report.Fields, B., Martin, M.A.& Kamley, D. (ed.), 22:319-328 (1985), Cold SpringHarbor Laboratory Press, Cold SpringHarbor]. moreover, [Oncogene, 4:759-766(1989)]. which is 4-5 mg/l in P210 BCR-ABL — if a recombination transition vector including the 5 'PORIHE drine compounds 5 without deficit united with RBcDNA which removed most non-coding fields' non-translated field is used, high RB protein manifestation level can be made still higher. This RBmRNA array is G+C Rich highly, and is a factor advantageous to forming stable secondary structure. These structures are considered to make translation effectiveness of Correspondence mRNA low when it is before an initiation codon. about the in vitro translation of RBmRNA, it proves [become / 10 times / high] from 5 times by permuting RB5' a non-translated array by it of an alfalfa mosaic virus (AMV) RNA 4 or beta globulin mRNA — having — this — RB5' — [EMBO J. (1990)] which is what suggests having a bad influence on the translation of a non-coding array potentially. Moreover, if 5' non-translated array with a long foreign gene exists, it will also have become clear that the recombination PORIHE drine compounds manifestation in the Baculoviridae system is influenced. since A+T rich nature of a polyhedrin promotor is very strong, before inserting in a transition vector as a conclusion — long — and G+C — rich 5' non-coding array should be trimmed from RBcDNA, and the pp110RB manifestation should be optimized.

[0090] In order to suppress the denaturation of the protein under purification to the minimum, several kinds of different protocols were tested about elution of RB protein from an affinity column. Since many are not known so much about the biological functions and the biochemical property of RB protein, it is only two parameters that it can be used as a scale of the integrity of purification protein. That is, it is complex formation with DNA avidity and an SV40 T antigen. In addition, the conditions which maintain a proteinic biochemical property were elution conditions which use the triethylamine of 20mM(s) by pH10.8 in this invention. Although purification protein carried out the nuclear normal position immediately from the cytoplasm after the microinjection, it is proved [this / protein / this] that activity is shown under these elution conditions. When protein was eluted by extreme pH (triethylamine of the glycine of 200mM, pH 2 and 3, or 100mM(s), pH11.5), the inclination for protein to denaturalize was accepted. That is, the two above-mentioned activity fell. This is clear also from insoluble floc having formed after prolonged preservation.

[0091] According to the former report, although [non-phospho RURIRU-ized RB protein] the SV40 T antigen of D2C2 cell which is the stable transformant by the SV40 T antigen of nephrocyte network valve flow coefficient1-P of an ape is combinable, it was found out that the HIPOHOSUHORIRU-ized gestalt of [Cell, 56:57-65 (1989)] and RB protein of a certain kind can also form an SV40 T antigen and complex. This has been checked with sufficient repeatability, when in vitro mixing of the purification RB protein [from a T antigen and an AcNPV-Y4RB infection

insect cell] or malt -4 lysate was carried out. The same phenomenon was accepted also when complex was formed by in vivo one using a Cos cell (drawing 10). Since phosphoryl-ization of RB protein is changed in a cell cycle in the case of phase singularity and the complex formation between RB and viral neoplasm protein is participating in the transformation activity of these DNA tumor virus, the relevance of HIPOHOSUHORIRU-ized RB protein and an SV40 T antigen should be solved someday.

[0092] RB protein which does not have a meltable deficit when the Baculoviridae insect cell lineage is used, as explained above and which can also predict activity suddenly — an owner — it is a big advance for future biochemical and research of a biophysical property of RB gene production object that it can be used in a meaningless amount. They are analysis of the cell protein in relation to the case considered, three-dimension-structure research of RB protein using separation and X-ray crystallography of the specific DNA array with which these interact, etc. Moreover, the elucidation of the biological functions of the retinoblastoma gene in cancer control should also progress. About the intervention to the cell growth of RB and differentiation which were directly tested by the microinjection considered, it is inquiring on the current energy target.

[0093] The notation used on these specifications is explained below. cDNA — complementary DNA and kd — KIRODARUTON and kb — a kilobase and SDS — for Nonidet P-40 and MES, sodium salt (2-[N-morpholino] ethane sulfonic acid) and MOI are [a sodium dodecyl sulfate and PAGE / polyacrylamide gel electrophoresis and NP-40 / relative molecular weight and PAP of the multiplicity of infection and Mr] potato acid phosphatase. The protein production object shown by "pp110RB" is the same protein production object as "ppRB110."

[0094] It is included by the pneuma and the range which various kinds of modification is possible, and all indicated to the claim although this invention has been explained about the specific example. That is, this indication does not mean a limit.

[Translation done.]

* NOTICES *

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

- [Drawing 1] It is the schematic diagram showing the configuration of a Baculoviridae manifestation vector which carries out pp110RB composition.
- [Drawing 2] It is the western blotting of a ppRB infection insect cell.
- [Drawing 3] It is the western blotting which identifies the cell extract extracted from the infected cell even 72 hours after infection.
- [Drawing 4] It is the microphotography in which the normal position between cells of RB protein is shown.
- [Drawing 5] It is the microphotography in which infection Sf9 cell is shown.
- [Drawing 6] It is the radioautograph which shows phosphoryl-izing of RB protein and the dephosphorylation analysis result in an insect cell.
- [Drawing 7] pMG 3-245 -- anti- -- the electrophoresis analysis of the rough lysate from -RB, infection Sf9 cell, and eluate is shown.
- [Drawing 8] The fusion protein and the south Western DNA binding measuring method of Baculoviridae manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10% are shown.
- [Drawing 9] It is the radioautograph of the blot from parallel gel to the gel used in order to create drawing 8 , and the incubation of this blot is carried out by the 32P-labeling DNA fragment.
- [Drawing 10] It is the chromatogram which shows complex formation with Baculoviridae manifestation RB protein and an SV40 T antigen.
- [Drawing 11] It is the photograph in which the purification RB protein nucleus normal position after the microinjection of cell ***** of Saos-2 cell is shown.

[Translation done.]

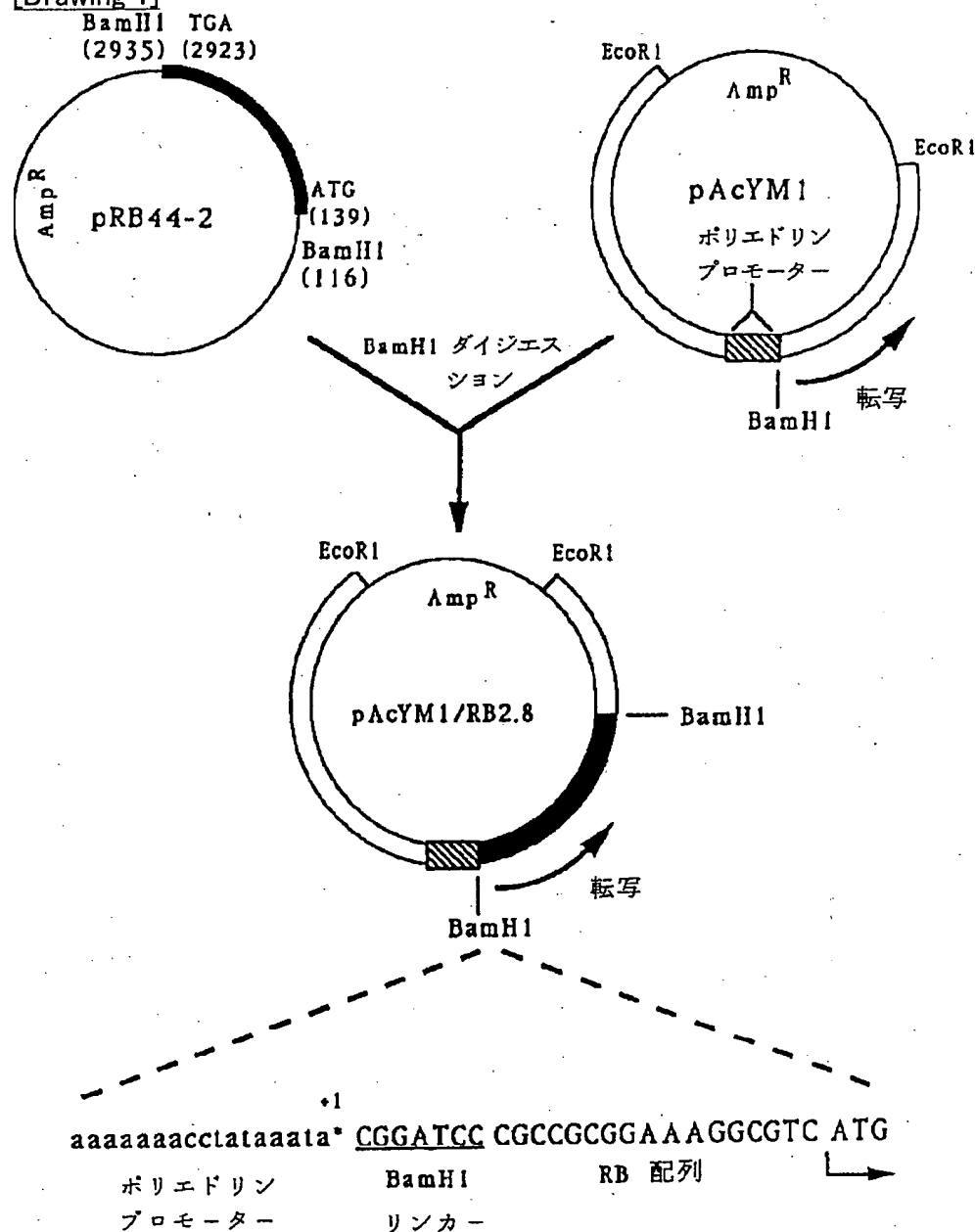
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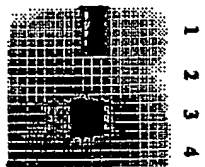
DRAWINGS

[Drawing 1]



[Drawing 2]

pp10 RB
p10 RB



図面代用写真

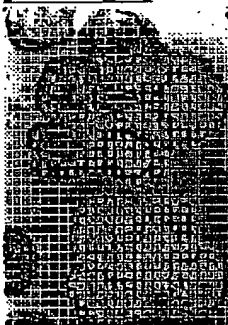
[Drawing 3]

pp10 RB
p10 RB



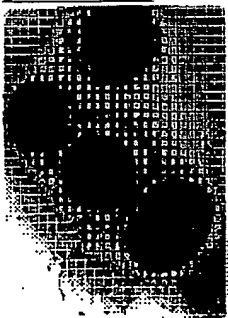
図面代用写真

[Drawing 4]



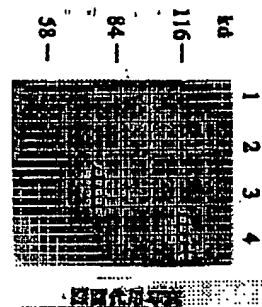
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[Drawing 5]



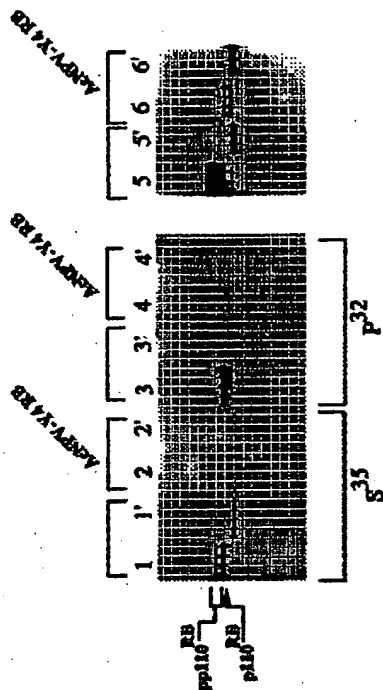
図面代用写真

[Drawing 8]



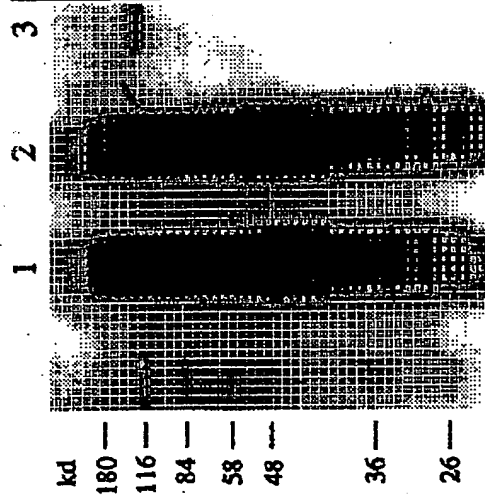
写真

[Drawing 6]



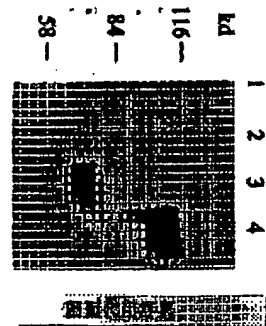
写真

[Drawing 7]

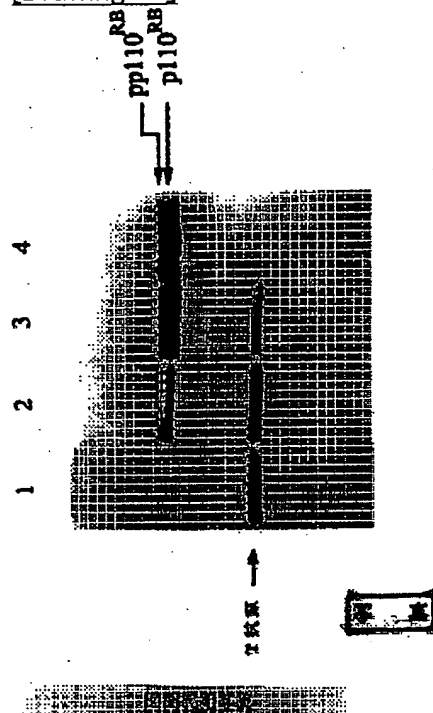


写真

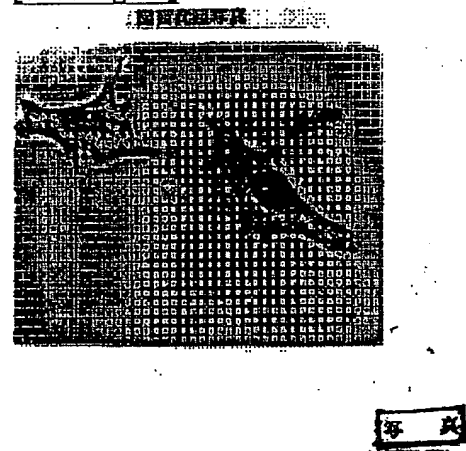
[Drawing 9]



[Drawing 10]



[Drawing 11]



[Translation done.]

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WRITTEN AMENDMENT

[Procedure revision]

[Filing Date] August 18, Heisei 5

[Procedure amendment 1]

[Document to be Amended] Specification

[Item(s) to be Amended] Easy explanation of a drawing

[Method of Amendment] Modification

[Proposed Amendment]

[Brief Description of the Drawings]

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最終頁に続く

(54)【発明の名称】 遺伝子タンパク質産生物及び細胞治療法

(57)【目的】 遺伝子タンパク質産生物の産生方法の提供。

【構成】 遺伝物質の昆虫細胞への導入によって所望ポリペプチドを実質的な量で産生する方法。適当なベクターを使用して、例えば、クローン化遺伝子、遺伝子フラグメント、あるいはその誘導体を宿主細胞に導入することによって高純度のタンパク質を実質的な量で、高レベルで産生できる。

【特許請求の範囲】

【請求項1】 所定の遺伝子に関係する実質的に精製された遺伝子産生ポリペプチドを産生する方法において、該遺伝子にヌクレオチド配列に実質的に対応するヌクレオチド配列をもつcDNAを搬送するベクターを選択し、

該DNAを該ベクターに挿入し、

該ベクターを細胞培養物に導入して、該ポリペプチドを伝搬する発現系を形成し、

該細胞を成長させ、

該細胞を分裂させ、

該細胞から該ポリペプチドを抽出し、そして該ポリペプチドを精製することからなるポリペプチド産生方法。

【請求項2】 該ベクターがウィルスである請求項1に記載の方法。

【請求項3】 該ウィルスがバキュロウイルスである請求項1に記載の方法。

【請求項4】 該ウィルスがAutographa californica核多角体病ウィルスである請求項1に記載の方法。

【請求項5】 該cDNAがポリヘドリン遺伝子プロモーター特性をもつ請求項1に記載の方法。

【請求項6】 該cDNAがポリヘドリン遺伝子のプロモーターをもち、そして該クローン化cDNAを該プロモーターの下流に挿入する請求項1に記載の方法。

【請求項7】 該細胞が真核生物細胞である請求項1に記載の方法。

【請求項8】 該細胞がSpodoptera frugiperda細胞である請求項1に記載の方法。

【請求項9】 該精製を免疫親和クロマトグラフィー法によって実施する請求項1に記載の方法。

【請求項10】 該cDNAを真核生物遺伝子から誘導する請求項1に記載の方法。

【請求項11】 該cDNAを真核生物癌抑制遺伝子から誘導する請求項1に記載の方法。

【請求項12】 該cDNAを網膜芽腫遺伝子から誘導する請求項1に記載の方法。

【請求項13】 所定の遺伝子に関係する遺伝子産生ポリペプチドを発現するポリペプチド発現系において、搬送用ベクターと細胞培養物からなるポリペプチド発現系。

【請求項14】 該ベクターがウィルスである請求項13に記載の発現系。

【請求項15】 該ベクターがバキュロウイルスである請求項13に記載の発現系。

【請求項16】 該ベクターがAutographa californica核多角体病ウィルスである請求項1に記載の系。

【請求項17】 該細胞培養物の細胞が昆虫細胞である請求項13に記載の系。

【請求項18】 該細胞がSpodoptera frugiperda細胞である請求項13に記載の系。

【請求項19】 所定の遺伝子に関係し、かつ該遺伝子にヌクレオチド配列に実質的に対応するヌクレオチド配列をもつcDNAを搬送するベクターを選択し、

該DNAを該ベクターに挿入し、

該ベクターを細胞培養物に導入して、該ポリペプチドを伝搬する発現系を形成し、

該細胞を成長させ、

10 該細胞を分裂させ、

該細胞から該ポリペプチドを抽出し、そして該ポリペプチドを精製することからなる方法によって得た実質的に精製されたポリペプチド。

【請求項20】 網膜芽腫遺伝子から該cDNAを誘導することを含む請求項19に記載のポリペプチド。

【請求項21】 欠損癌抑制遺伝子、突然変異性癌抑制遺伝子をもつ、あるいは遺伝子をもたない細胞を治療する方法において、

該欠損あるいは突然変異性遺伝子を、あるいは遺伝子のないことを確認し、

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該遺伝子の、天然産生遺伝子又はそのクローンである欠損のない対部分を求め、そして対部分の遺伝子のタンパク質産生物を産生することからなる治療方法。

【請求項22】 該搬送が該タンパク質産生物を該細胞に顕微注射することを含む請求項21に記載の方法。

【請求項23】 該精製が該タンパク質の緩衝液への透析を含む請求項21に記載の方法。

【請求項24】 該緩衝液がトリス-HCl、KCl、EDTA、DTT及びグリセロールを含む請求項21に記載の方法。

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【請求項25】 該トリス-HClのpHが7.4である請求項24に記載の方法。

【請求項26】 該緩衝液が1部のDTT、1部のEDTA、100部のKCl及び200部のトリス-HClを含む請求項23に記載の方法。

【請求項27】 該確認がタンパク質産生物の有無を調べ、かつタンパク質産生量を測定する請求項21項に記載の方法。

【請求項28】 該測定が該タンパク質に特異的な抗体を使用して、該抗体と該タンパク質の免疫複合体が形成するかどうかを決定することを含む請求項21に記載の方法。

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【請求項29】 活性成分としてのポリペプチドと生理学的に適当な担体とからなる薬剤組成物。

【請求項30】 該ポリペプチドがpp110^{RS}である請求項29に記載の薬剤組成物。

【請求項31】 乳癌抑制遺伝子、ヴィルムス腫瘍抑制遺伝子、ベックウィズ-ヴィーデマン症候群抑制遺伝子、遷移性細胞腫瘍抑制遺伝子、神経芽腫抑制遺伝子、小細胞肺癌抑制遺伝子、腎細胞腫抑制遺伝子及び結腸直

50

腸癌抑制遺伝子のタンパク質産生物から該活性成分を選択する請求項29に記載の薬剤組成物。

つ請求項29に記載の薬剤組成物。

【化1】

【請求項32】 該活性成分の以下のアミノ酸配列をも

(34)
(74)
(114)
(154)
(194)
(234)
(274)
(314)
(354)
(394)
(434)
(474)
(514)
(554)
(594)
(634)
(674)
(714)
(754)
(794)
(834)
(874)
(914)
(928)

E A S K S L I V Q D V S F E L S P
P R M K V M I E T S A K N L R L A T N P
D E E L L K P R P E S A K N L R L A T N P
E R D L L P T L F S A Q D E P Q S I S K I P M
E V L R V S D G S N F I T L C T L G D Y R K R
P M D S L L N N D L K S G H A E L Y V P S P T
P D V M A K E S I I E L S K A R M E S R P R
P P A A S I L T S M K R D I E N E S E R P N T
P I A N N F Q V D M F E L M L A C C E P T S S
P K I D I Y K L T L I E N E H T L M K I N G T
P L F V E D A G Q Q Y E Q R D S T M I H K E M
P K I K T L I L Q G S S T T N N I L P T A E
A Q C T S V R S T I I K S T L P V L Q V I P S A
P C I S T S C A N K T I I K S T L P V L Q V I P S A
P L G T S L S M D N K M R G E T L L K S P K K
E A W D S M R F H M V S S E R T Y H F L L L Q
A T L I S L M P D V R E Y A D S A R T T G V Q
A F E E P Q Q I L T K M T K K G L D E P E R F
A D K K Q F G F F R L V A I S K R R Q P S D K
A P K L T S R N L V I R M F Q K Y M V R I S S
A E K L L I R K R P S Y V S K K V L A T K N E
A E Q N Y V P F A T E Y V E I P K E H S Y C G
T T I F I L T Y D H K L E I L S K Y P A P V P
A E Y F L D R V L P P R L V D R Y E L Y S M L
A E G K E D P N D P N V A K F V F N D Q K Q N
T F G H C E S K K I V G C Y L P L Q K L L N K
R K E L V T M G V N V T L A F P S L S L Y I P I S K G E K
P R L V S R Q N E K N C K L D S S L S T A N S P I S K G E K
T V D E I L E P I Y E N R S A S M T Q V T I K Y F A K
P K L V I K E I N I E F Q M K L D S T F I L I K K E N
P P S N S G V C E D Y S N L W A K L I R I K K E D S
P L S K F K A E E L S G F N A A L T K Q G S S T
M D V Q L A T H E Y N I I L L T P W F M G T G D
E K L A L K E R S L E N V S H K I K F P G E M
P E E F L Y K K K N V D N E N Q I L V I F I S
G W T L F P C S R E C N L M N T M D S R S D D
S T F V T E L L P S G L I I Q Q E I N N L E F N
D L T D I K V N T P Q L W R L F L N Y P G R M
Q W F Y W L E E R Q G K P H P A E K F S I L K

【発明の詳細な説明】

【0001】

【産業上の利用分野】 本発明は全体的には遺伝子タンパク質産生物の産生方法及び細胞を治療して腫瘍形成を抑制するための細胞療法に関する。本発明はまた細胞療法及び腫瘍形成を抑制する細胞治療法に関する。本発明は

政府助成（認可番号EY05758）発明であり、全国保険協会（the National Institute of Health）及びカルフォルニア大学と共同して達成した発明である。従って、本発明については政府にも一定の権利がある。

【0002】

【従来技術及びその課題】ヒトや動物の組織についてはかなり詳しい研究があり、数多くの欠損が細胞レベルで確認されている。これら欠損の多くは遺伝的な原因をもつもので、大半が欠損遺伝子によるものである。欠損は点突然変異等を原因とすることがあり、この結果遺伝子自体のヌクレオチド配列が分断したり、異常な変化を示すことがある。機能不全遺伝子があると、最終的には遺伝子タンパク質産生物を産生できなくなるか、それ自体が欠損した遺伝子タンパク質産生物を産生することになる。

【0003】ヒト・動物のいずれについても欠損遺伝子を同定する場合には、遺伝子療法を適用することができる。この場合には、クローン化遺伝子を治療すべき細胞の細胞核に導き、異常な遺伝物質または欠損遺伝物質を矯正すればよい。この方法に使用される物質は製造コストが高く、複雑精巧な実験装置を必要とし、また複雑精巧な遺伝子操作技術の適用を必要とする。このような技術は一般には利用できず、従って利用できるのは大部分が比較的少数の高度に精巧な分子遺伝子操作を扱う実験室に限られている。

【0004】遺伝子療法は費用が高く、一般に利用できないため、別な細胞療法が求められている。分子構造の分析及びタンパク質産生物の機能から、該産生物を産生する遺伝子の保険に関して結論を導くことは可能である。例えば人体の腫瘍抑制機序を決定するためには、多くの場合遺伝子タンパク質産生物を使用して、遺伝子間の相互作用を決定すればよい。腫瘍抑制機序に関しては、本願の親特許出願明細書を参照すればよい。遺伝子機能及び遺伝子相互作用を解明するためには、信頼性が高く、またコストが低い上に、信頼性高くかつ予測可能に多量のタンパク質を提供できる遺伝子タンパク質産生物を産生する方法が非常に望ましい。

【0005】遺伝子タンパク質産生物は遺伝子機能及び遺伝子相互作用を解明するのに有用だけでなく、該タンパク質自体が欠損遺伝子状態の治療に使用できる。この場合、欠損遺伝物質をもつ細胞に適当な遺伝子タンパク質産生物を導入することが有利であり、効果的である。場合によって、タンパク質産生物の導入の方が、遺伝物質自体を治療を目的として投与する場合よりもコストが低く、容易なことがある。

【0006】タンパク質療法については、本願と同時に提出する特許出願明細書を参照すればよい。

【0007】遺伝子タンパク質産生物の重要性に関する認識からみた場合、実質的に精製された形で遺伝子タンパク質産生物を調製・分離する方法が利用できるようになるのが非常に有利である。生化学的に活性な物質を多量にそのまま利用できることは、遺伝機序に関与している生化学的特性及び分子挙動を研究するためにだけでなく、治療のためにも有意義な前進である。

【0008】一般に、大量産生の場合と異なる実験室規

模の産生では、遺伝子タンパク質産生物は細胞からだけでなく、その合成産生によっても産生している。細胞からの誘導についていえば、細胞タンパク質はごく少量存在しているに過ぎない。この結果、天然源から十分多量のタンパク質を誘導する試みは現実的ではない。

【0009】合成産生法についていえば、遺伝子の解読配列を細菌発現ベクターに導入してタンパク質を発現する試みは一部の場合を除いて成功していない。細菌産生タンパク質は溶解性が弱い。細菌発現システムを使用する別な欠点は、細菌細胞が真核生物タンパク質を変更できず、またタンパク質について翻訳後変更が必要な場合には、このようなタンパク質の分析が誤った結果を与える点にある。つまり、細菌産生タンパク質は一般に溶解性が低く、分子欠損があるため、有用性に制限がある。

【0010】タンパク質産生物を作る通常の実験室的方法では、十分に多量のタンパク質を産生できないだけでなく、産生タンパク質の純度も十分でなく、純度にバラツキがある。ある種のタンパク質産生物を産生するさいの難しさの代表例には、TrpE-RB融合タンパク質を発現し、そしてE. coliに発現するT7RNAポリメラーゼを使用して、ポリペプチドを産生することがある。これら方法は比較的複雑であり、複雑精巧な生化学的操作を必要とする。さらに、これら方法には、目的のポリペプチドがごく少量しか産生しないので、大きな制限がある。また、この方法で産生したポリペプチドは分子的にみて好ましくないことが多い。というのは、例えば、これはホスホリル化しないからである。

【0011】従って、遺伝子産生物ポリペプチドの重要性から、所望の生化学的及び生物物理学的特性をもつポリペプチドを実質的な量で産生する方法が強く望まれている。

【0012】生体健康維持における遺伝子の機能の理解については、かなりの進歩がみられる。一般的にいえることは、細胞遺伝子が適正なタンパク質を産生できないと、生体に各種の異常が発生する。この産生できない理由は遺伝子全体が失われているか、あるいは遺伝子自体が各種の原因で欠損していることに求めることができる。これら因子の認識から、遺伝子療法にかなりの進展が認められる。

【0013】例えば、幼児の腎臓癌であるヴィルムス腫瘍は染色体11の遺伝子不活性化によって生じると考えられている。一本鎖染色体の微小細胞融合媒介転移法を使用した場合には、正常な染色体11をヴィルムス腫瘍細胞に導入すると、腫瘍形成性を抑制できることが実証されている。一方、染色体X及び13の導入にはこの効果は認められない。

【0014】実験レベルでは、欠損のないヒト染色体の転移はある程度は有用であるが、このような転移は遺伝子欠損の治療には利用できない。ひとつには、治療に好適な染色体の調製はかなりの熟練を必要とするだけでな

く、時間がかかる上にコストが高いからである。このため、この方法は多くの場合に許容できない。

【0015】欠損のない染色体を治療に利用するのが好ましくないとした場合、次に理論的に考えられることは、適正な遺伝子全体を、あるいは少なくともその効力のある部分を患者に導入することである。この方法は欠損のない染色体を導入する方法よりも実行可能性が高いが、遺伝子療法が望まれているのはある一定の場合のみである。

【0016】この点に関して、適正な核酸物質の分離、配列決定、クローニングは非常にコストが高いだけでなく、時間がかかるものである。加えて、このような方法では、世界でもごく限られた場所でしか利用できない複雑精巧な分子遺伝子操作方法が必要である。さらに、現在でも、このような方法は治療に適する多量の物質を産生するようになっていない。

【0017】以上から、生物工学的方法を利用し、そして比較的成本が低く、信頼性があり、より一般的に利用でき、かつ生化学的作用において特異的な物質を使用する細胞レベルの特定療法が強く望まれている。さらに、細胞レベルで治療に有効な産生物を導入して、腫瘍抑制等の変化をもたらす療法も強く望まれている。いうまでもないことだが、バラツキのない精製状態で多量に産生でき、そして容易にかつ有効に欠損細胞に導入できる産生物についても強く望まれている。

【0018】本発明の第1目的は癌抑制を制御するのに有効な、全体的に安全でかつ特異的な療法及び産生物を提供することにある。

【0019】本発明の別な目的は生物工学的方法及び産生物を利用する、癌腫瘍の抑制及び根絶に有効な癌抑制制御産生物及び療法を提供することにある。また、細胞レベル及び細胞間レベルで作用を発揮する、癌治療用薬剤組成物を提供することも本発明の目的である。

【0020】本発明のさらに別な目的は天然又は合成産生物を活性成分とする、欠損又は突然変異癌抑制遺伝子や、癌抑制遺伝子の不在によって生じる症状を治療する薬剤組成物を提供することにある。

【0021】本発明は特異的癌抑制遺伝子タンパク質産生物を対象細胞に導き、癌抑制を実現する細胞療法からなる。

【0022】本発明は従来の放射線療法や化学療法の必要性が少ない、癌の治療法を提供するものである。さらに、本発明方法は遺伝的に癌に罹患しやすい素因の発見後、腫瘍形成が開始する前の極めて早い時期に適用することが可能である。

【0023】本発明は癌抑制遺伝子タンパク質産生物を好便にかつ比較的低いコストで使用して細胞レベルで癌抑制を実現する点において極めて有利である。

【0024】ある細胞中のひとつかそれ以上の癌抑制遺伝子が不活性化した場合、それで十分癌を発生すること

になるかについてははっきりとしていない点があるが、遺伝子タンパク質産生物の細胞導入は新しいアプローチであり、悪性腫瘍治療への有利なアプローチである。本発明は従来からの細胞毒性癌療法とは違い、生体への損傷を最小限に抑えた状態で、細胞レベルで有利な変化をもたらす点においてさらに有利である。

【0025】上述したように、本発明の第1目的は実質的な量の欠損のない、活性遺伝子産生ポリペプチドを産生する方法を提供することにある。

【0026】更に本発明の別な目的は構造及び機能が天然産生ポリペプチドと同じである、特異的遺伝子産生ポリペプチドの産生方法を提供することにある。

【0027】要約すれば、本発明の上記目的及びほかの目的は遺伝物質を昆虫細胞に導入することによって実質的な量の所望ポリペプチドを産生する方法により実現できる。例えば、クローニング遺伝子や遺伝子フラグメント又はその誘導体を適当なベクターを利用して、宿主細胞に導入すれば、高純度のタンパク質を実質的な量でかつ高レベルで産生することができる。

【0028】本発明は高品質のポリペプチドを実質的な量で産生して、細胞レベルで遺伝子機能を調べることできる方法を提供するもので、極めて有利なものである。

【0029】また、本発明はこのポリペプチドを有利で信頼性があり、しかも反復的に、比較的低いコストで産生できるもので、この点においても非常に有利であり。

【0030】さらに、本発明は細胞レベル及び細胞間レベルで遺伝子間相互作用を解明できる、相当量の高品質ポリペプチドを産生できるもので、この点でも有利である。

【0031】

〔図面の簡単な説明〕

本発明の上記上記目的及び特徴、並びにほかの目的及び特徴、そしてこれらの実現方法は以下の記載から明らかになるはずである。また、本発明それ自体の理解は添付図面に参考にして、発明の実施例に関する記載を読めば得られるはずである。図1はpp110RDを合成するバキュロウィルス発現ベクターの構成を示す概略図である。

図2はppRB感染昆虫細胞のウエスタンブロット(Western blot)である。図3は感染から72時間後までに感染細胞から抽出した細胞抽出物を示すウエスタンブロットである。図4はRBタンパク質の細胞間定位を示す顕微鏡写真である。図5は感染Sf9細胞の顕微鏡写真である。図6は昆虫細胞におけるRBタンパク質のホスホリル化及び脱ホスホリル化分析の結果を示すラジオオートグラフである。図7はpMG3-245抗-RBからの粗溶解産物、感染Sf9細胞及び溶解物の電気泳動分析結果を示す図である。図8は10% SDS-PAGE、クマシーブリリアントブルー染色法に適用した融合タンパク質及びバキュロウィルス発現pp110RDのサウスウエスタン(Southwes

tern) DNA結合測定法を示す。図9は図8を作成するために使用したゲルに対する平行ゲルからのプロットのラジオオートグラフであり、このプロットは³²P-標識化DNAフラグメントでインキュベーションしたものである。図10バキュロウィルス-発現RBタンパク質とSV40T抗原との錯体形成を示すクロマトグラムである。図11Saos-2細胞の細胞質への顕微注射後における精製RBタンパク質の核転移を示す。

【0032】

【発明の最良の実施例】本明細書で言及する文献はいずれもその内容をここに取り入れてあり、本明細書記載の一部を構成している。以下、次の順序で本発明を説明することにする。

A. 概説

B. RB遺伝子産生物例

B1. 組換えバキュロウィルスの構成

B2. 感染昆虫細胞における外性RBタンパク質の発現

B3. 外性RBタンパク質の核定位及び翻訳後のホスホリル化

B4. 感染昆虫細胞からのRBタンパク質精製

B5. DNA-結合活性及びSV40T抗原との特異的複合体形成

B6. 精製RBタンパク質の核転移

B7. 要約

【0033】A. 概説

本発明は昆虫細胞培養物に遺伝物質を導入することによって遺伝子タンパク質を産生する方法に関し、この遺伝子タンパク質は培養によって特異的な遺伝子タンパク質産生物を産生するのに使用できる。本方法を使用すれば、力値の高い、実質的に精製された、欠損のない、生化学的に活性なタンパク質を得ることができる。

【0034】本発明の発現系は広い用途をもつ。すなわち、例えば、ヒトや動物のクローン化遺伝子やそのフラグメント、相同体、誘導体あるいは部分は所望タンパク質産生物の産生に利用することができる。このようにして産生したタンパク質はいうまでもなく欠損細胞の治療に、遺伝子機能の解明に使用できる。というのは、遺伝子は細胞レベルで相互作用するからである。

【0035】遺伝子タンパク質産生物を産生する場合、昆虫細胞培養物は真核生物特性をもつので、好適であることが見いだされた。遺伝物質を細胞培養物に導入するためにはウィルスベクター等の通常のベクターを使用することができる。

【0036】例えば、RB遺伝子タンパク質産生物を産生するためには、昆虫細胞培養物のウィルスベクターを使用する。この点に関して、培養昆虫細胞において高レベルで組換えタンパク質を産生するためには、ヘルパーに依存しないウィルス発現ベクターとしてバキュロウィルスのオートグラフ・カリホルニカ (*Autographa californica*)、即ち核多角体病ウ

イルス (AcNPV) を使用する。このウィルスは培養したアメリカ産行列毛虫ヨトウガ、即ちスポドプテラ・フルギベルダ (*Spodoptera frugiperda*) (Sf9) 細胞中で繁殖する。このウィルスは多角体遺伝子の強力な、一時的に調節されたプロモーターをもつものであり、その産生物は溶解感染時に全細胞タンパク質の50%以上を占める。インビボ組換えにより、外来遺伝子の解読配列はポリヘドリンプロモーターの翻訳制御を受け、この結果高レベルでタンパク質を発現する。さらに、このようにして産生したタンパク質は正確に倍加し、元の高級真核生物に見られるタンパク質と同様な翻訳後変更を含んでいる。

【0037】本発明は特異的な癌抑制遺伝子タンパク質産生物を対象細胞に導入して、腫瘍抑制を実現する細胞療法である。所与の細胞が、欠損遺伝子をもつため、細胞におけるタンパク質発現欠損を招く点において欠損していることがある。

【0038】本発明方法は欠損遺伝子に関係する遺伝子タンパク質産生物を使用することに関する。精製タンパク質産生物を対象細胞に導入して、例えば、腫瘍抑制を実現する。この産生物を薬理学的に好適な担体に担持して導入することにより、該タンパク質産生物が細胞レベルで、あるいは細胞下レベルで作用する。

【0039】この方法の一例として、癌抑制遺伝子であるRB遺伝子が欠損しているか、あるいはそれがない細胞RB遺伝子タンパク質をに導入した。RB遺伝子に関する詳しい情報については、本明細書で言及している親特許出願明細書を参照すればよい。

【0040】好適な実施例では、劣性腫瘍形成研究のプロトタイプモデルとして網膜芽腫、即ち発育中の網膜の稀な症状である、幼児癌を使用する。染色体13q14に対する、関与している遺伝要素の定位及びその劣性性に基づき、癌抑制遺伝子と推定されている遺伝子、即ち網膜芽腫感受性遺伝子 (RB) をクローン化した。この遺伝子は27のエクソンが200キロ塩基のゲノムDNAに分散し、検査した正常な組織にすべてに4.7キロ塩基mRNA転写を発現する。相補DNAクローンの配列分析により、928のアミノ酸の仮説的タンパク質をコード化できた長いオープンリーディングフレームが明らかになった。RB cDNA配列から推測した所定エピトープに対する抗体を使用したところ、このRB遺伝子産生物は相対分子量 (Mr) が110,000~114,000の核リンタンパク質と同定された。これを p p110^{RB} と命名した。

【0041】網膜芽腫にだけではなく、RB遺伝子機能の欠損はまた乳癌、骨肉腫、前立腺癌や小細胞肺癌を始めとする他の幾つかの腫瘍にも関与していることが判明している。最近の研究によれば、レトロウィルス媒介遺伝子転移により網膜芽腫、骨肉腫や前立腺癌細胞にRB遺伝子を再導入すると、ヌードマウスにおける腫瘍形

成を始めとする新生表現型の幾つかの面を明らかに抑制することが判明している。これはRB遺伝子の腫瘍抑制機能の直接的な証拠である。ところが、この生物学的活性の分子レベルにおける基礎はまだ確立されていない。

【0042】現在までのところ、癌抑制遺伝子産生物の生化学的特性及び生物学的機能はいずれも解明されていないが、これはこのタンパク質が細胞中に少ないため、十分な量で得ることが難しいからである。この点に関しては、本願と同時に出願した親出願明細書（発明の名称：遺伝子タンパク質産生物の産生方法）を参照すればよい。

【0043】欠損遺伝子をもつ、あるいは遺伝子のない細胞に特異的遺伝子タンパク質産生物を導入する細胞療法を発明した。本発明によれば、実質的に精製された、欠損のない、生化学的に活性な遺伝子産生タンパク質の適正な量を治療上有効な投与量で欠損遺伝子に導入できる。

【0044】本発明の細胞療法は適用用途が広い。本タンパク質産生物は欠損細胞の治療だけでなく、遺伝子機能の解明にも有用である。というのは、遺伝子が細胞レベルで相互作用するからである。

【0045】ひとつの特定実施例では、網膜芽腫遺伝子タンパク質産生物、即ちpp110^{RB}は欠損遺伝子をもつ、あるいは遺伝子のない真核生物細胞の治療に広く適用できる。

【0046】なお、本発明によれば、真正なヒトpp110^{RB}と全く同様に、精製タンパク質がDNAを結合し、SV40T抗原と特異的錯体を形成できた。また、顕微注射後直ちに行ったタンパク質の核転移によると、このタンパク質が活性を示し、治療に使用できることが判明した。

【0047】B. RB遺伝子産生物

次に、pp110^{RB}タンパク質の本発明による産生方法についてさらに詳しく説明する。このタンパク質及び網膜芽腫遺伝子に関するさらに詳細な情報については、以下の親特許出願明細書を参照すればよい。

【0048】RB遺伝子等の癌抑制遺伝子産生物の生化学的特性及び生物学的機能はいずれも解明されていないが、これは精製タンパク質を十分な量で得ることが難しいからである。即ち、ひとつには、タンパク質が細胞に少ないからであり、また加えて、遺伝子のコード化配列の細菌発現ベクターへの導入によるタンパク質発現が一部を除いて成功していないからである。これらから、現在利用されている方法がもつ問題は真核生物系にクローン化遺伝子を発現すれば避けることができると結論できた。本発明の特定実施例はRB遺伝子タンパク質産生物の産生に関するが、本発明は、例示すれば癌抑制遺伝子であるが、とはいってもこれに制限されない他の真核生物遺伝子のタンパク質産生物の産生にも適用でき、従ってこのようなタンパク質産生物の産生にも関するもので

ある。

【0049】培養昆虫細胞において高レベルで組換タンパク質を産生する為には、ヘルパーに依存しないウィルス発現ベクターとしてバキュロウィルスのオートグラフア・カリホルニカ (*Autographa californica*)、即ち核多角体病ウィルス (AcNPV) を使用するが好ましい。このウィルスは培養したアメリカ産行列毛虫ヨトウガ、即ちスポドプテラ・フルギペルダ (*Spodoptera frugiperda*) (Sf9) 細胞中で繁殖する。このウィルスは多角体遺伝子の強力な、一時的に調節されたプロモーターをもつものであり、その産生物は溶菌感染時に全細胞タンパク質の50%以上を占める。インビボ組換により、外来遺伝子の解読配列はポリヘドリンプロモーターの翻訳制御を受け、この結果高レベルでタンパク質を発現する。さらに、このようにして産生したタンパク質は正確に倍加し、元の高級真核生物に見られるタンパク質と同様な翻訳後変更を含んでいる。

【0050】バキュロウィルス系により機能性RBタンパク質の発現可能性をテストする為には、RB遺伝子の完全なコード化配列を含むクローン化ヒトRB cDNAをAcNPV発現ベクターに導入し組換えウィルスを昆虫細胞中で繁殖させた。ホストベクター系により高レベルでヒトpp110^{RB}発現を実現できた。得られたタンパク質をホスホリル化し、感染細胞の核を正確に標的とした。さらに、RBタンパク質の精製法も確立した。なお、本発明によれば、真正なヒトpp110^{RB}と全く同様に、精製タンパク質がDNAを結合し、SV40T抗原と特異的複合体を形成できた。また、顕微注射後直ちに行ったタンパク質の核転移によると、このタンパク質が活性を示し、治療に使用できることが判明した。

【0051】B1. 組換えバキュロウィルスの構成
バキュロウィルス発現系においてRBタンパク質を最大限に産生するために、RB遺伝子から大部分の5'非コード化配列を取り除くことにより組換え転移ベクターを構成した。座特異性突然変異誘発によって、RB cDNAのヌクレチオド116及び2935に2つのBamH1座を導入して、組換え転移ベクターの構成を促進した。図1に示すように、精製したpAcYM1/RB2.8は、完全な(60塩基対)ポリヘドリン5'非コード化配列がRB cDNAの5'末翻訳領域に融合し、その後完全なコード化配列が続くmRNAをコード化する。この組換え遺伝子は真正なRB開始座のヌクレチオド139の上流にATGコドンを含まない。従って、組換え遺伝子は非融合、フルレンジRBタンパク質をコード化する。

【0052】図1に転移ベクターpAcYM1を示すが、これらはすべて開始ATGコドンのAを含むポリヘドリン遺伝子の上流配列をもち、この後に特徴的なBamH1座が続く。転移ベクターについては、松浦等の記

載がある。J. Gen. Virol., 68:1233-1250 (1987)。pRB44-2はプラスマ pGEM1 (Promega) の BamH1 座にサブクローン化したヌクレオチド116から2935にいたる完全な RBcDNA コード化配列を含む。組換えバキュロウィルスベクター、即ち pAcYM1/RB2. 8 については、2.8 kb の BamH1 フラグメントを pRB44-2 から適正な配向方向に pAcYM1 の BamH1 座に挿入して構成したため、RB 遺伝子の転写をポリヘドリンプロモーターの直接制御下におくことができた。

【0053】バキュロウィルス発現ベクターを構成して pp110^{RB} を合成するさいには、以下の点を考慮した。p^{RB} 44-2 は pGEM1 の BamH1 座にサブクローン化したヌクレオチド116から2935にいたる完全な RBcDNA コード化配列からなる。pAcYM1 はポリヘドリン遺伝子の側面に位置するウィルス DNA 配列の約 7 kb の EcoR1 フラグメントを含み、この場合先導配列はそのままであるが、ATG の第 1 A を除くポリヘドリンコード化配列はすべて BamH1 リンカーによって置換する。ポリヘドリンプロモーター RBcDNA 融合を含む組換えバキュロウィルスベクター、すなわち pAcYM1/RB2. 8 については、2.8 kb の BamH1 フラグメントを pRB44-2 から適正な配向方向に pAcYM1 の BamH1 座に挿入して構成したため、RB 遺伝子の転写をポリヘドリンプロモーターの直接制御下におくことができた。図 1 の下の方に、融合接合部の配列を示す。また、図 1 の下のケース記号はポリヘドリンプロモーターを、上のケース記号は RBcDNA 配列を示す。そして BamH1 リンカーにはアンダーラインしてある。RB の ATG (ヌクレオチド 139) を利用する融合遺伝子の翻訳は矢印で示す。図 1 の a* (+1) はポリヘドリン遺伝子の翻訳開始コドン ATG の第 1 A を示す。

【0054】RBcDNA の組換えプラスミドからウィルスゲノムへの転写は、リポフェクション (lipofection) により野生型 Autographa californica 核多角体病ウィルス DNA を用いて PaCym1/RB2. 8 DNA をコントランスフェクション (cotransfection) することにより行った (BRL)。同族組換えにより RB 遺伝子とのアレリック置換によりポリヘドリン遺伝子を不活性化した組換えウィルスは、感染細胞にポリヘドリン閉鎖体が認められなかったため、そのプラーク形態によって同定した。ウィルスを 3 回プラーク精製して、純粋なバキュロウィルス株を得た。これを AcNPV-Y4RB とした。

【0055】B2. 感染昆虫細胞における外生 RB タンパク質の発現

AcNPV ポリヘドリンプロモーターが異種無脊椎動物

細胞にヒト RB 遺伝子の発現をもたらすかどうかを決定する前に、Sf9 細胞を用意し、3.33 gm/l のイーストレート (yeastolate)、ラクトアルブミン加水分解産物 (GIBCO) 及び 10% の加熱不活性化ウシ胎児血清 (GIMINI 社) を加えたグレースの昆虫培地 (Grace's insect medium) - Bull. 1555, (1987) (テキサス農業実験所、カレッジ実験所、TX) を用いて、27℃で単層又は懸濁培養により Sf9、即ち Spodoptera frugiperda IPLB-Sf21-AE [In vitro, 13:213-217 (1977)] を培養した。細胞溶解産物を大規模産生するさいには、Sf9 細胞のスピンナー培養物を血清を含まない EX-CELL 400 規定培地 (J. R. Scientific 社) で培養した。ヒト T 白血病細胞系統のモルト (Molt) - 4 細胞を 20% ウシ血清を加えた RPMI 1640 培地で懸濁培養した。骨肉腫細胞系統の Saos-2 細胞を 7.5% ウシ胎児血清を加えたダルベッコ (Dulbecco) 変性イーグル培地で培養した。

【0056】AcNPV ポリヘドリンプロモーターが異種無脊椎動物細胞にヒト RB 遺伝子の発現をもたらすかどうかを決定するさいには、プラーク精製 AcNPV-Y4RB で Sf9 細胞を感染処理した。感染 40 時間後に、感染細胞の溶解産物を集め、抗-RBO-47 抗原で免疫沈降処理した。次に、サンプルを SDS-PAGE 処理してから、ウェスタンブロット分析した。

【0057】図 2 及び図 3 に、ウェスタンブロット分析により行った AcNPV-Y4RB 感染昆虫細胞における pp110^{RB} 同定結果を示す。図 2 の場合、感染 40 時間後に、模擬感染細胞 (経路 2)、AcNPV-Y4RB 感染細胞 (経路 3)、あるいは野生型 AcNPV 感染 Sf9 細胞 (経路 4) から細胞抽出物を調製した。ヒト白血病細胞系統のモルト-4 を対照として使用した (経路 1)。

【0058】図 3 に示すように、単クローン系抗体で免疫ブロット処理した場合、AcNPV-Y4RB 感染細胞の抽出物 (経路 3) においては哺乳動物細胞 (経路 1) と同様のフルレングスの RB タンパク質を発現が認められたが、模擬感染細胞や野生型 AcNPV 感染細胞 (経路 2、4) の場合には認められなかった。図 3 の場合には、RB タンパク質産生の最適時期を求めるために、感染後時間をかけて、AcNPV-Y4 感染細胞から細胞抽出物を得た。溶解産物を抗-RBO-47 抗原で免疫沈降処理し、pMG3-245 単クローン系抗体で免疫ブロット処理した。図 3 の pp110^{RB} 及び pp110^{RB} はそれぞれ未ホスホリル化タンパク質及びホスホリル化タンパク質を表す。感染後は、RB タンパク質の産生をモニターして、細胞を回収する最適な時期を求めた。図 3 に示すように、RB タンパク質産生は感染 24

時間後に検出でき、続く12時間の間に産生量はかなり増加した。タンパク質産生レベルは感染後約72時間にわたりずっと維持し、そして72時間後に細胞の溶菌が実質的に開始した。細胞溶解に伴うタンパク質劣化を最小限に抑えるために、通常、感染から約40時間後に、感染細胞を回収した。

【0059】RBタンパク質の発現を検出する際には、AcNPV-Y4RBを使用して、0.5のMOIでSf9細胞を感染処理した。感染からそれぞれ24、36、48、60及び72時間後に、 5×10^4 の細胞を1mlの溶解緩衝液(50mMのNaCl、pH7.4; 0.2%のノニデット(Nonidet)P-40; 1mMのEDTA; 100mMのNaCl; 50mMのNaF及び1mMのPMSF)中で溶解し、溶解産物を5分間遠心処理して清澄化した。溶解産物を次に抗-RB0.47抗原でインキュベーションして、免疫沈降物を7.5%SDS-PAGEで分離した。この後、タンパク質をニトロセルロース紙に移し、常法で処理した。一夜遮断状態で放置した後、3時間ニトロセルロース紙をpMG3-245抗-fRB単クローン系抗原でインキュベーション処理してから、Cell, 54:275-283(1988)の記載に従ってアルカリ性ホスファターゼ接合ヤギ抗-マウスIgG及び発色基質で処理した。

【0060】B3. 外生RBタンパク質の核定位及び翻訳後のホスホリル化

RB遺伝子は分子量(Mr)が110,000の核リンタンパク質をコード化する。バキュロウィルスにより昆虫細胞に産生したRBタンパク質が核に正確に到達したかを調べるために、感染から40時間後に抗-RB0.47抗原を用いてAcNPV-Y4RB-感染Sf9細胞を免疫染色した。免疫染色により昆虫細胞に発現したRBタンパク質の細胞間定位を図4及び図5に示す。図4には模擬感染Sf9細胞の場合を、そして図5にAcNPV-Y4RB-感染Sf9細胞の場合を示す。図4及び図5に示すように、感染細胞は異常に大きい核を含んでいた。このような状態はバキュロウィルス感染の細胞変性効果に特有なものである。模擬感染又は野生型AcNPV感染Sf9細胞を抗-RB0.47抗原でインキュベーションしたところ、染色は認められなかった

(図4)。ところが、AcNPV-Y4RB感染細胞の核にのみ強い染色が認められた(図5)。AcNPV-Y4RB感染Sf9細胞からの核及び細胞形質抽出物をSDS-PAGE及びウエスタンブロッティングにより分析したところ、外生RBタンパク質は主に核部分に存在することが確認できた。

【0061】免疫染色は次の工程で行った。模擬感染、野生型AcNPV感染、AcNPV-Y4感染のいずれも場合も感染から40時間後にSf9細胞をポリ-L-リシン(Sigma社)をコーティングしたチャンバース

ライド(Miles Scientific社)に接種し、一夜インキュベーションした。いずれも次の工程に移る前にスライドをリン酸緩衝塩化ナトリウム水溶液で洗浄した。即ち、まづ4%ホルムアルデヒドの0.04Mリン酸緩衝液を用いて20分間、あるいはアセトン(-20℃)を用いて10分間細胞を固定した。2%の正常なヤギ血清のPBS溶液で10分間固定細胞を前インキュベーションしてから、0.02%のトリトンX-100に希釈した抗-RB0.47抗原で一夜インキュベーションした。洗浄後、ビオチニル化したヤギ抗-ウサギIgG(カナダ、バーリンゲームのTAGO社)を加えた。一時間後、セイヨウワサビペルオキシダーゼ(カナダ、バーリンゲームのVector Labs社)で接合したAB複合体を用いて45分間細胞をインキュベーションしてから、次の基質を用いてインキュベーションした。この基質は0.05%の3,3'-ジアミノベンジジンテトラヒドロクロリド、及び0.01%のH₂O₂の0.05Mトリス-HCl溶液から構成した。pH7.6(Sigma社)。3~5分後に細胞をPBS洗浄することにより反応を停止した。次に、ニコ製のダイアフォトマイクロスコープ(diaphotomicroscope)で細胞の写真を取った。

【0062】図6に、昆虫細胞に産生したRBタンパク質のホスホリル化及び脱ホスホリル化の結果を示す。AcNPV-Y4RB感染から40時間後に、³⁵S-メチオニンあるいは³²P-オルトリン酸を用いて3時間でSf9細胞を代謝標識化した。対照としてモルト-4を使用し、抗-RB0.47抗原を用いて細胞溶解産物を免疫沈降した。ポテト酸ホスファターゼ(PAP)処理する前に(経路1、2、3、4)、あるいは処理後に(経路1'、2'、3'、4')SDS-PAGEにより³⁵S-及び³²P-標識化RBタンパク質免疫複合体を分離し、ラジオオートグラフィーにより分析した。同様に、未標識化細胞からの溶解産物を使用して脱ホスホリル化実験を行い、ポテト酸ホスファターゼ処理前後(経路5、6及び5'、6')にウエスタンブロット分析を行った。

【0063】さらに続けて図4の場合を説明する。RBタンパク質のホスホリル化は多重セリン及びトレオニン残基で生じるもので、SDS-PAGEにおけるRBタンパク質の分子量不均一性を説明するものである。[Oncogene Res., 1:205-214(1989); Cell, 56:57-65(1989)]昆虫細胞に産生したRBタンパク質が翻訳後にホスホリル化を受けるかどうかを調べるために、感染後3時間から40時間で³⁵S-メチオニンあるいは³²P-オルトリン酸を用いてAcNPV-Y4RB-感染Sf9細胞を代謝標識化した。細胞抽出物を免疫沈降処理し、SDS-PAGE分析してから、ラジオオートグラフィー処理した。この点については、図6の経路2及び4をそれぞれ

参照すればよい。これと平行して、同じ抽出物からの免疫沈降性RBタンパク質をポテト酸ホスファターゼ(PAP)で処理して、SDS-PAGEにおけるRBタンパク質移動性への脱ホスホリル化効果をテストした。脱ホスホリル化後、³⁵S-標識化RBタンパク質の分子量110,000は2重バンドから1重バンドに落ちた(図6の経路2')。そして³²P-標識化RBタンパク質から殆ど完全に放射能が放出した(図6の経路4')。AcNPV-Y4RBで感染処理した、未標識化細胞からの溶解産物についてのウエスタンブロッティングによる脱ホスホリル化分析も、PAP処理と同じバンド変化パターンを示した(図6の経路6、6')。これら観察結果から、昆虫細胞に産生したRBタンパク質はホスホリル化していたことがわかった。また、この変化からSDS-PAGEで認められたRBタンパク質の分子量不均一性を説明できた。

【0064】Sf9昆虫細胞の放射線標識化及び脱ホスホリル化分析は次に示す工程で実施した。感染から40時間後に、10%のウシ胎児血清を加えた、メチオニン、リン酸のいずれも含まないDME培地で60mmの皿を用いて30分間Sf9細胞(3×10⁶)をインキュベーションした。この後、3時間で0.25mCi/mlの³⁵S-メチオニン(1134Ci/mole、NEN)か0.25mCi/mlの³²P-オルトリン酸(担体なし、ICN)のいずれかを加えることにより細胞を代謝標識化した。溶解緩衝液(50mMのトリス-

HCl、pH7.4; 0.2%のノニデットP-40; 1mMのEDTA; 100mMのNaCl; 50mMのNaF及び1mMのPMSF)を用いて細胞を抽出し、抽出物を抗-RB0.47抗原で免疫沈降処理した。

【0065】³⁵S-又は³²P-標識化細胞溶解産物、及び未標識化細胞溶解産物からの免疫沈降RBタンパク質の2/3をポテト酸ホスファターゼ(PAP、Boehringer社)脱ホスホリル化分析した。[Oncogene Res.、1:205-214(1989)]。反応緩衝液(20mMのMES、pH5.5; 100mMのNaCl; 1mMのMgCl₂; 50μMのロイペプチン)37℃、60分間で1.5単位のPAPを用いて、RBタンパク質含有免疫複合体をインキュベーションし、反応後、RBタンパク質を7.5%SDS-PAGE分析してから、ラジオオートグラフィーかウエスタンブロッティングにより分析した。

【0066】B4. 感染昆虫細胞からのRBタンパク質の精製

1. 0の感染多重度(MOI)でAcNPV-Y4RBによりSf9細胞を感染処理し、感染から40時間後に細胞溶解産物を得た。この条件でバキュロウィルス系に発現したRBタンパク質の全レベルは感染昆虫細胞培養物1リットルにつき約17~18mgであった("10⁹細胞)。この点については、表1を参照すればよい。

【0067】

【表1】

表1

バキュロウィルス感染昆虫細胞からの組換えRBタンパク質の精製

工程	合計タン パク質量 (mg)	RBタン パク質量 (mg)	収率 (%)	精製度	純度 (%)
細胞抽出 pMG3-245	670 ^a	16 ^c	90 ^c	1.0倍	2.3
免疫親和カラム	13.5 ^b	12.9 ^d	72 ^{cd}	41.3倍	95

a. ブラッドフォード(Bradford)(Bio-RAD社)の方法によるタンパク質量

b. マイクロBCA(PIERCE社)及び分光測光法によるタンパク質量

c. ウエスタンブロット法及び濃度測定法によるタンパク質量

d. クマシーブリリアントブルー染色法及び濃度測定法によるタンパク質量

【0068】表1に示すように、発現したRBタンパク質の90%(16mg)は細胞分裂後の上澄みに認められ、10%が不溶画分に止まっていた。全細胞タンパク質の2.3%を占めていたので、RBタンパク質は細胞溶解産物から簡単に検出できた。1段免疫親和クロマトグラフィー精製後、約135mgのタンパク質をカラムのアルカリ性溶離物から回収できた。溶離RBタンパク質の純度を調べるために、2.5×10⁵細胞に相当す

る溶離物アリコットをSDS-PAGE及びクマシーブリリアントブルー染色により分析した。

【0069】この点に関しては、pp110^{ab}の免疫親和クロマトグラフィー精製を示す図7を参照すればよい。

1×10⁵の、模擬感染Sf9細胞(経路1)又はAcNPV-Y4RB感染Sf9細胞(経路2)からの粗溶解産物、及びpMG3-245抗-RB免疫親和クロマトグラフィーから溶離物のアリコット(2.5×10⁵感染細胞に相当)を10%SDS-ポリアクリロアミドゲルによる電気泳動法によって分析してから、クマシーブリリアントブルー染色法により分析した。予想分子量のRBタンパク質は矢印で示してある。

【0070】濃度測定法により判断すると、上記1段精製工程は有効であり、RBタンパク質を純度95%(図7、経路3)、収率72%、そして精製度41.3倍で得ることができた(表1)。

【0071】免疫親和カラムはシュナイダー等や島西等の記載に従って構成したが、若干の変更を加えた。

[J. Biol. Chem.、257:10766-10769 (1982); Virology、144:88-100 (1985)]。バイオラッド (Bio-Rad) カラムに2mlのタンパク質G-アガロース (Genex社) を充填し、0.01NのHClで洗浄した後、結合緩衝液 (0.1Mの酢酸ナトリウム、pH5.0; 0.1MのNaCl) で洗浄した。カラムに2回15mgの抗-fRB単クローン性抗原 (pMG3-245) を加え、結合を行った。次に、カラムを0.1Mの硼酸緩衝液で広く洗浄し、ビーズを20mlの緩衝液に再懸濁した。最終濃度が40mMになるようにジメチルピメリデートジヒドロクロリド (Sigma社) を加え、混合物を室温で1時間攪拌し、架橋反応を行った。洗浄後、室温で10分間、40mMのエタノールアミン-HClの0.1M硼酸緩衝液20ml溶液、pH8.0、でビーズの残留反応性基を遮断した。この後、0.2Mのグリシン、pH2.3、でカラムを洗浄し、トリス緩衝液 (50mMのトリス-HCl、pH7.4; 100mMのNaCl; 1mMのPMSF; 1mMのEDTA) で中和し、必要になるまでこの溶液に保存した。最初の単クローン性抗原サンプルのOD₂₈₀及び次の工程における流動画分のOD₂₈₀を測定したところ、約10mgのpMG3-245が2mlのタンパク質G-アガロースに結合していた。

【0072】B5. DNA結合活性及びSV40T抗原との特異的複合体形成

現在のところ、RBタンパク質については2つの生化学的特性が記述されている。即ち、固有な性質としてDNAを結合できることがそのひとつであり [Nature、329:642-645 (1987)]、そして何種類かのDNA腫瘍ウィルスの腫瘍タンパク質と特異的複合体を形成できることがもうひとつである [Cell、54:275-283、(1988); Science、243:934-937 (1989); Nature (London)、334:124-129 (1988)]。タンパク質の生物学的機能に關与しているこれら2つの公知生化学的特性について、バキュロウィルス感染昆虫細胞から精製したRBタンパク質をテストした。

【0073】図8及び図9にサウスウエスタンDNA-結合測定法を示す。6μgの精製trpE-RB融合タンパク質、及び精製バキュロウィルス発現pp110^{RB}を10%SDS-PAGEに適用した。図8に示した結合測定法の場合には、クマシーブリリアントブルー染色法を使用し、図9に示した結合測定法の場合には、ニトロセルロース紙に平行ゲルを電気転写した。プロットを次に³²P-標識化DNA画分でインキュベーションし、ラジオオートグラフィー分析した。図8及び図9の場合

次の通りである。経路1:RB19-22;経路:RB23-27;経路3:RB19-27;経路4:AcNPV-Y4RB感染昆虫細胞からの精製RBタンパク質。

【0074】図8及び図9の場合、DNA結合をサウスウエスタン分析により測定した。この場合、同量のtrpE-RB融合タンパク質、及び昆虫細胞からの精製RBタンパク質を10%SDS-PAGEにより分離した。ローディングされたタンパク質の量はクマシーブリリアントブルー染色法によって求めた (図8)。別な平行ゲルを使用した実験では、ニトロセルロース膜に電気転写してから、³²P-標識化DNAでインキュベーションした。タンパク質に結合したDNAを次にラジオオートグラフィーにより分析した (図9)。その主ドメインがDNAと相互作用する融合タンパク質RB19-27のDNAに対する親和力は2つの領域RB19-22及びRB23-27よりも20倍高い。この点については、図9の経路3は経路1及び2に比較でき、精製フルレングスRBタンパク質はRB19-27と同様に強いDNA結合活性を示した (図9、経路4)。昆虫細胞からの精製RBタンパク質のDNA結合活性はDNAセルロースによるタンパク質の保持によっても、またカラムからのその後に続く溶離一約400mMのNaCl一によっても証明できた。

【0075】感染昆虫細胞からのpp110^{RB}の精製は次のようにして実施した。1.0のMOIでAcNPV-Y4RBを用いてSf9細胞を感染処理し、懸濁培養した (1X10⁶細胞/ml、1000ml)。感染から40時間後に、低速遠心分離によって細胞をペレット化し、洗浄し、そして50mMのトリス-HCl、pH7.4; 0.2%のNP-40; 1mMのEDTA; 100mMのNaCl; 10%の (v/v) グリセロール; 1mMのDTT; 1mMのPMSF; 25μgのロイペプチン及び50単位/mlのアプロチニンを含む抽出緩衝液に再懸濁した。氷上で15分間インキュベーションした後、サンプルを遠心 (10,000xg、4℃、10分間) 清澄化処理し、RB含有上澄みを回収した。前述したように、タンパク質G-アガロースに結合した抗-fRB単クローン性抗原 (pMG3-245) を含む2ml容量のカラムでpp110^{RB}の免疫親和クロマトグラフィーを実施した。上澄みをカラムに4回通した後、それぞれ次の溶液を200ベッド容量の量で用いてカラムを連続的に洗浄した。溶解緩衝液、500mMのNaClを含む溶解緩衝液及び洗浄液 (200mMのNaCl; 1mMのEDTA; 1mMのDTT; 1mMのPMSF; 10%のグリセロール)。次に、2mMのトリエチルアミン、pH10.8; 200mMのNaCl; 1mMのEDTA; 1mMのPMSF及び10%のグリセロールを含むアルカリ性溶離緩衝液で結合タンパク質をカラムから溶離した。1ml画分を回収し、直

ちに1Mのトリス-HCl (pH7.5) の1/12容量で中和し、10%グリセロール中に-70℃で保存した。

【0076】感染昆虫細胞からpp110^{RB}を精製するさいに、全タンパク質量を求めてから、サウスウエスタンDNA結合測定法及びSV40T抗原結合測定法を実施した。

【0077】免疫親和カラムの溶離画分の全タンパク質の量をマイクロBCA測定法(PIERCE)により求めた。溶離タンパク質サンプルを次にSDS-PAGEにより分析し、そして溶離物のRBタンパク質の量をクマシーブリリアントブルー染色法、次に濃度測定法により求めた。細胞抽出物の全タンパク質量はブラッドフォードの方法(Bio-Rad)により測定した。[Anal. Biochem.、72:248-254(1976)]。細胞溶解産物のRBタンパク質を定量するために、標準として順に希釈した精製RBタンパク質を使用して、ウエスタンブロッティングを実施してから、バンド強度について濃度測定により比較した。この点については、表1を参照すればよい。

【0078】常法によりタンパク質ブロッティングを実施した。ボウエン(Bowen)等の記載に従ってプロットを放射線標識化DNAによりインキュベーションした。[Nucleic Acids Res. 8:1-21(1980)]。これは室温で行った。プロットを素早く水洗してから、6Mの尿素及び0.2%のNP-40(いずれも20分)で3度洗浄し、引き続き、DNA結合緩衝液(10mMのトリス-HCl、pH7.0; 1mMのEDTA; 50mMのNaCl; 0.2%のBSA; 0.2%のFicoll 400及び0.2%のポリビニルピロリン)で4回洗浄(いずれも30分)した。次に、³²P-標識化DNAを含むDNA結合緩衝液中で30分間プロットをインキュベーションした。EcoRIで線状化したpGEM1DNAをランダムプライミングにより α -³²P-デオキシヌクレオチド(Amersham、>3000Ci/mmol)で標識化し、プローブとして使用した。交雑後、プロットをDNA結合緩衝液で3回(いずれも10分)洗浄し、風乾し、ラジオオートグラフィーによって分析した。対照としてTrepE-RB融合タンパク質を含めた。タンパク質が含むRB遺伝子のエクソンに従い各trepE-RB融合タンパク質を命名した。すなわち、RB19-22、RB23-27及びRB19-27はそれぞれエクソン19-22(アミノ酸612-775)、エクソン23-27(アミノ酸776-928)及びエクソン19-27(アミノ酸612-928)のpp110^{RB}領域を占める。

【0079】Ad-SVX1-感染293細胞からSV40T抗原を免疫クロマトグラフィー精製した。[J. Virol.、53:1001-1004(198

5); Cold Spring Harbor Press.、Cold Spring Harbor, NY、pp. 187-192(1982)]。そして、抗-T単クローン性PAG419抗原をOncogene社から得た。公知複合体形成測定法を実施した。但し、若干の変更を加えた。即ち、800ngのバキュロウイルス発現RBタンパク質を1mMのPMSF、25 μ g/mlのロイペプチン及び50単位/mlのアプロチニンを含む1mlのEBD緩衝液(50mMのトリス-HCl、pH8.0; 120mMのNaCl及び0.5%のノニデットP-40)と混合した。混合物に800ngの精製Tを加え、90分間氷上でインキュベーションした。抗-RB0.47又はPAB419抗原のいずれかを用いて混合物のアリコットを免疫沈降し、ウエスタンブロッティング分析した。プロットは順にpMG3-245、PAB419と反応させた。アルカリ性ホスファターゼ接合ヤギ抗-マウスIgGでインキュベーションした後、発色性基質を用いてプロットを発色処理した。

【0080】精製RBタンパク質がSV40T抗原と特異的複合体を形成できる能力をテストするために、等量のRBタンパク質とT抗原を混合し、抗-RB0.47抗体か抗-T抗原PAB419のいずれかを用いて、混合物のアリコットを免疫沈降した。

【0081】この点に関連して、図10にバキュロウイルス発現RBタンパク質とSV40T抗原とが形成する複合体を示す。インビトロで精製バキュロウイルス発現RBタンパク質を精製T抗原と混合した。次に、同アリコットの混合物をPAB419(経路2)か抗-RB0.47(経路3)のいずれかで免疫沈降し、ウエスタンブロッティングにより分析した。経路1及び4はそれぞれPAB419で免疫沈降した精製SV40T抗原、及び抗-RB0.47抗体で免疫沈降した精製バキュロウイルス発現RBタンパク質を示す。

【0082】図10に示すように、インビトロでRBタンパク質とT抗原を混合すると、RBタンパク質とPAB419が共免疫沈降するだけでなく(経路2)、Tと抗-RB0.47抗原が共免疫沈降した(経路3)。これらデータはバキュロウイルス感染昆虫細胞からのRBタンパク質がSV40T抗原と特異的複合体を形成できることを実証している。

【0083】B6. 精製RBタンパク質の核転移
精製タンパク質がインビトロでRBの2つの公知生化学的特性をもつことが判明したので、次に精製タンパク質のインビトロ挙動について調べた。エクソン21-27のない、欠損RB遺伝子を含み、かつC末端切断RBタンパク質(p95)をコード化する骨肉腫細胞系とのSaos-2細胞の細胞形質に精製RBタンパク質を注射した。[Proc. Natl. Acad. Sci. U. S. A.、87:6-10(1990)]。抗体はRBタンパク質のC末端に向かう事実を考慮して、使用する

抗-RB0.47抗体によって認められない程度の少量で細胞形質にこのタンパク質を定位した。注射直後に、細胞を固定し、免疫染色法によって分析した。図11にSaos-2細胞の細胞形質へ顕微注射した後の生成RBタンパク質の核定位を示す。細胞に生成RBタンパク質を注射し、免疫染色法により分析した。図中、矢印は非注射細胞に比べて、顕微注射後の核の染色が強いことを示している。

【0084】図11に示すように、非注射細胞に比べて、顕微注射後の核の染色が強く(矢印)、注射タンパク質が核に直ちに輸送されたことを示す。RBタンパク質は核タンパク質として知られているので、顕微注射後、精製タンパク質の核定位が直ちに、そして正確におきるが、これはこのタンパク質がインビボで活性なことを示唆している。

【0085】顕微注射の場合、20mMのトリス-HCl、pH7.4; 10mMのKCl; 0.1mMのEDTA; 0.1mMのDTT及び2%のグリセロールを含む注射緩衝液に最終濃度が0.5mg/mlになるように精製RBタンパク質を透析した。ガラス毛管針(Eppendorf社)を使用して、ガラスチャンバースライド上に成長したSaos-2細胞を通常の方法により顕微注射した。真空加圧装置を取り付けたEppendorf社のマイクロマニピレーター及び逆相コントラスト顕微鏡(ニコン製)を使用して、それぞれ毛管を顕微操作し、そして顕微注射過程を視覚化した。顕微注射後、直ちに4%ホルムアルデヒドの0.04Mリン酸緩衝液溶液(pH7.4)で細胞を固定し、免疫染色法により分析した。

【0086】B6. 要約

以上説明したように、バキュロウィルスポリヘドリンプロモーターの転写制御の下ヒト網膜芽腫遺伝子産生物が効率的に発現できることが証明できた。RBタンパク質は細胞の成長を妨害するだけでなく、場合によってはそれに対して“毒性”を示す疑いがあったため、長い間、高いレベルでRBタンパク質を発現する試みは難しいと考えられていた。ポリヘドリンプロモーターからの外来遺伝子の転写は感染の後半で生じ、この後に細胞外ウィルス粒子の産生、そして細胞及び大半のウィルス遺伝子の遮断が続く。従って、過剰産生した場合細胞成長に有害なことがあるRBタンパク質等のタンパク質の合成にバキュロウィルス昆虫細胞系は有利である。この系の別な長所は昆虫及び哺乳動物細胞のタンパク質合成経路の類似性にある。

【0087】また、RBタンパク質は昆虫細胞の核に正確に到達することも判明し、これは哺乳動物の核定位信号が昆虫細胞により認識されることを含意している。バキュロウィルス発現系における組換えタンパク質のグリコシル化は高マンノース型のO-結合及びN-結合オリゴ糖に制限されているが、外来タンパク質の適当なホス

ホリル化はc-myc及びHTLV-I p40^xの発現について報告されている(J. Virol.)。RBタンパク質は以前からホスホリル化するが、グリコシル化はしないことが知られていた。これがバキュロウィルス発現系が機能性RBタンパク質の産生に好適な理由である。

【0088】本明細書に開示したように、感染昆虫細胞に産生したRBタンパク質は翻訳後にホスホリル化し、従って真正な哺乳動物のRBタンパク質の場合と全く同様に、ウェスタンブロットング分析によって多重バンドを分化できる。ところが、バンド強度からみれば、ハイパーホスホリル化RBタンパク質と比較した場合、未ホスホリル化及びホスホリル化形態が優勢である。現在までのところ、この現象がウィルス性溶解感染時の母集団の細胞周期状態の反映なのか、あるいは多量の外生RBが細胞に存在するため、昆虫キナーゼによるタンパク質のホスホリル化が単に不十分なためなのかは知られていない。ホスホリル化パターンが哺乳動物タンパク質の場合と正確に同じなのかを決定するためには、RBタンパク質のホスホリル化位置を精密にマッピングする必要がある。

【0089】バキュロウィルス系に発現した組換えRBタンパク質の全レベルは感染昆虫細胞培養物(10⁹細胞)1リットルにつき約17-18mgである。この発現レベルはこの系が発生する他の哺乳動物タンパク質に相当する。例えば、インターロイキン2の場合は10-20mg/lである[The Banbury Report. Fields, B., Martin, M. A. & Kamley, D. (ed.), 22:319-328 (1985), Cold Spring Harbor Laboratory Press, Cold Spring Harbor]。また、P210BCR-ABLの場合には、4-5mg/lである[Oncogene, 4:759-766 (1989)]。その5'非コード化領域の大部分を取り去ったRB cDNAに融合した、欠損のないポリヘドリン5'未翻訳領域を含む組換え転移ベクターを使用すれば、高RBタンパク質発現レベルを更に高くすることができる。このRB mRNA配列は高度にG+Cリッチで、安定な2次構造を形成するのに有利なファクターである。これら構造体については、開始コドンの前にある時、対応mRNAの翻訳効率を低くするものと考えられる。RB mRNAのインビトロ翻訳については、RB 5'未翻訳配列をアルファルファモザイクウィルス(AMV) RNA 4又はβ-グロブリンmRNAのそれと置換することにより5倍から10倍高くなることが実証され、これはRB 5'非コード化配列の翻訳に潜在的に悪影響を及ぼすことを示唆するものである[EMBO J. (1990)]。また、外来遺伝子の長い5'未翻訳配列が存在すると、バキュロウィルス系における組換えポリヘドリン発現に影響するこ

とも判明している。ポリヘドリンプロモーターはA+Tリッチ性が非常に強いので、結論としては、転移ベクターに挿入する前に、長く、かつG+Cリッチな5'非コード化配列をRB cDNAからトリミングして、pp110^{RB}発現を最適化すべきである。

【0090】精製中のタンパク質の変性を最小限に抑えるために、親和カラムからのRBタンパク質の溶離について何種類かの異なるプロトコルをテストした。RBタンパク質の生物学的機能及び生化学的特性についてはそれ程多くは知られていないので、精製タンパク質の完全性の尺度として使用できるのは2つのパラメーターのみである。即ち、DNA結合活性と、SV40T抗原との複合体形成である。なお、タンパク質の生化学的特性を維持する条件は、本発明ではpH10.8で20mMのトリエチルアミンを使用する溶離条件であった。顕微注射後に細胞形質から精製タンパク質が直ちに核定位したが、これは該タンパク質がこの溶離条件下で活性を示すことを実証するものである。極端なpH(2.00mMのグリシン、pH2.3、又は100mMのトリエチルアミン、pH11.5)でタンパク質を溶離すると、タンパク質が変性する傾向が認められた。即ち、上記2つの活性が低下した。これは、長期間保存後に、不溶な凝集体が形成したことからも明らかである。

【0091】以前の報告によれば、未ホスホリル化RBタンパク質のみがサル腎細胞系CV1-PのSV40T抗原による安定な形質転換株である、D2C2細胞のSV40T抗原を結合できるとされていたが[Cell, 56:57-65(1989)]、RBタンパク質のある種のヒポホスホリル化形態もSV40T抗原と複合体を形成できることが見いだされた。これは、T抗原とAcNPV-Y4RB感染昆虫細胞からの精製RBタンパク質、あるいはモルトー4溶解産物とをインビトロ混合した場合に再現性よく確認できた。同じ現象は、Cos細胞を使用してインビボで複合体を形成した場合にも認められた(図10)。RBタンパク質のホスホリル化は相特異性の場合細胞周期中に変動し、かつRBとウィルス性腫瘍タンパク質の間の複合体形成はこれらDNA腫瘍ウィルスの形質転換活性に関与しているので、ヒポホスホリル化RBタンパク質とSV40T抗原との関連性はいずれ解明すべきものである。

【0092】以上説明したように、バキュロウィルス昆虫細胞系を使用すると、可溶性、欠損のない、活性をもつと予測できるRBタンパク質を有意な量で使用できることは、RB遺伝子産物の生化学的及び生物物理学的特性の将来の研究にとって大きな前進である。考えられるケースには、関連する細胞タンパク質の分析、これらが相互作用する特異的DNA配列の分離やX線結晶学

を利用するRBタンパク質の3次元構造研究等である。また、癌抑制における網膜芽腫遺伝子の生物学的機能の解明も進展するはずである。顕微注射により直接テストした、RBの細胞成長及び分化への考えられる関与については、現在精力的に研究している。

【0093】本明細書で使用した記号について以下説明しておく。cDNAは相補DNA、kdはキロダルトン、kbはキロ塩基、SDSは硫酸ドデシルナトリウム、PAGEはポリアクリルアミドゲル電気泳動、NP-40はノニデットP-40、MESは(2-[N-モルホリノ]エタンスルホン酸)ナトリウム塩、MOIは感染の多重度、Mrは相対分子量、そしてPAPはポテト酸ホスファターゼである。“pp110^{RB}”で示したタンパク質産生物は“ppRB¹¹⁰”と同じタンパク質産生物である。

【0094】本発明を特定実施例について説明してきたが、各種の変更が可能であり、いずれも請求項に記載した精神及び範囲に包含されるものである。即ち、本開示は制限を意図するものではない。

【図面の簡単な説明】

【図1】pp110RB合成するバキュロウィルス発現ベクターの構成を示す概略図である。

【図2】ppRB感染昆虫細胞のウエスタンブロットである。

【図3】感染から72時間後までに感染細胞から抽出した細胞抽出物を同定するウエスタンブロットである。

【図4】RBタンパク質の細胞間定位を示す顕微鏡写真である。

【図5】感染Sf9細胞を示す顕微鏡写真である。

【図6】昆虫細胞におけるRBタンパク質のホスホリル化及び脱ホスホリル化分析結果を示すラジオオートグラフである。

【図7】pMG3-245抗-RBからの粗溶解産物、感染Sf9細胞及び溶解物の電気泳動分析を示す。

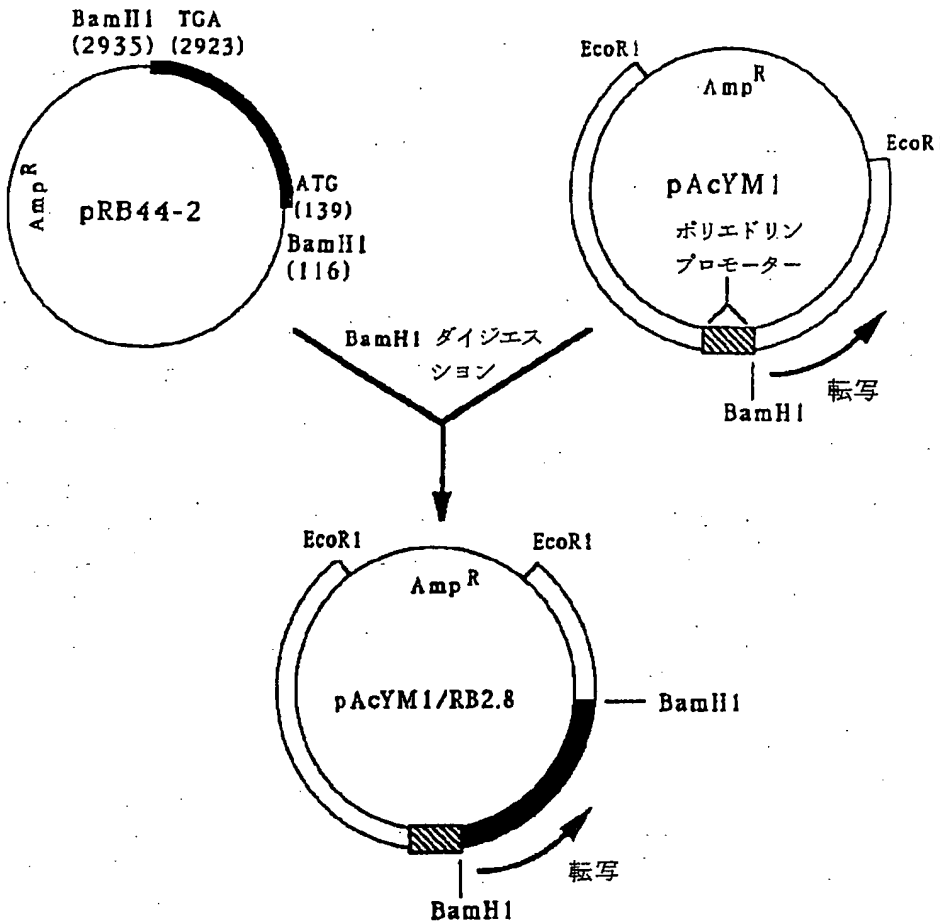
【図8】10%SDS-PAGE、クマシーブリアントブルー染色法に適用した融合タンパク質及びバキュロウィルス発現pp110RBのサウスウエスタンDNA結合測定法を示す。

【図9】図8を作成するために使用したゲルに対する平行ゲルからのプロットのラジオオートグラフであり、該プロットを32P-標識化DNAフラグメントでインキュベーションしたものである。

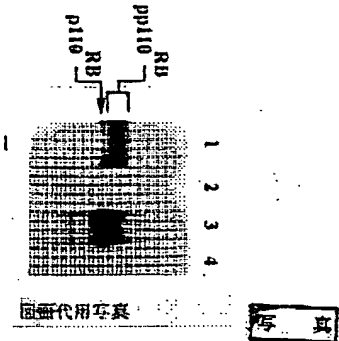
【図10】バキュロウィルス発現RBタンパク質とSV40T抗原との複合体形成を示すクロマトグラムである。

【図11】Saos-2細胞の細胞軽質への顕微注射後の精製RBタンパク質核定位を示す写真である。

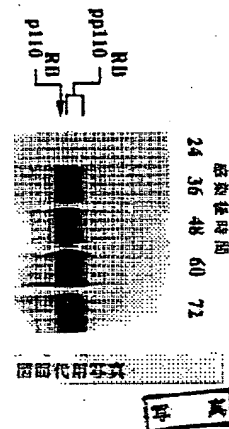
【図1】



【図2】

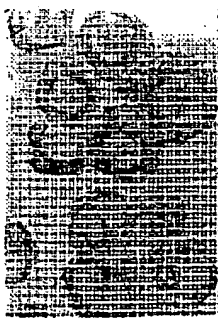


【図3】



+1
aaaaaaacclataaata* CGGATCC CGCCGCGGAAAGGCGTC ATG
ポリエドリン BamHI RB 配列
プロモーター リンカー

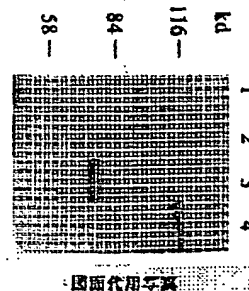
【図4】



【図5】

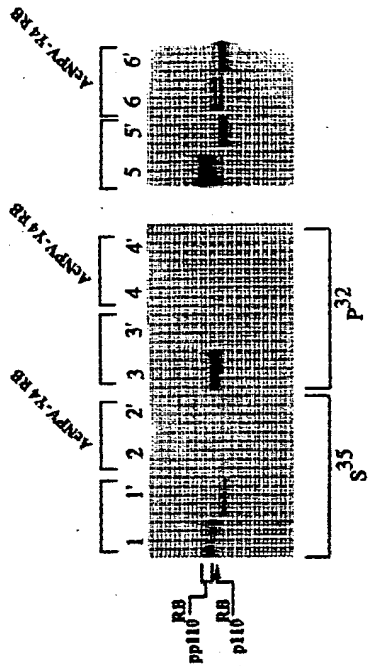


【図8】

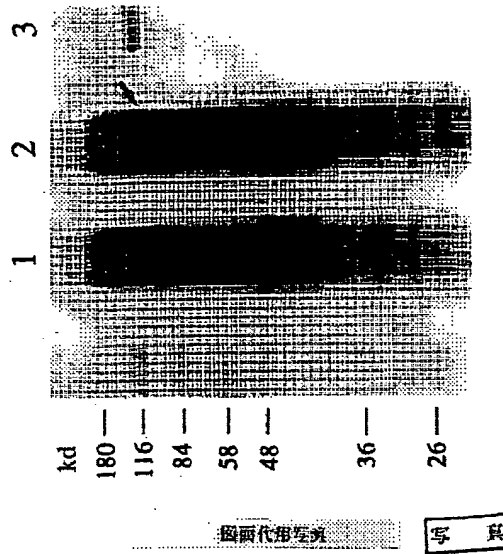


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【図6】



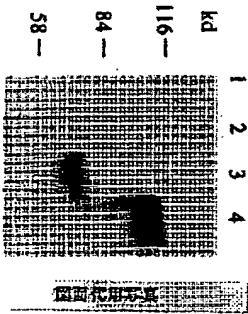
【図7】



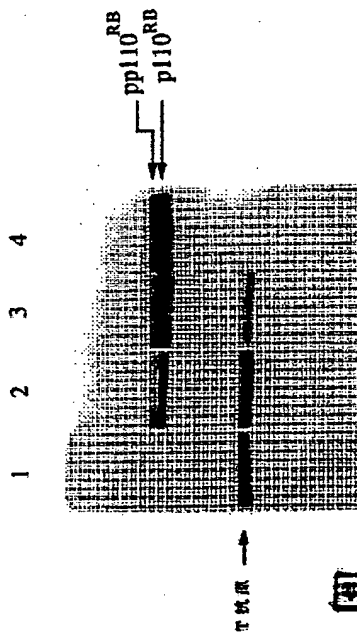
【図11】



【図9】



【図10】



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【手続補正書】

【提出日】平成5年8月18日

【手続補正1】

【補正対象書類名】明細書

【補正対象項目名】図面の簡単な説明

【補正方法】変更

【補正内容】

【図面の簡単な説明】

【図1】pp110RB合成するバキュロウィルス発現ベクターの構成を示す概略図である。

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化及び脱ホスホリル化分析結果を示すラジオオートグラフを示す写真である。

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